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Chevron

8EHQ-1197-13645

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CERTIFIED MAIL WITH RETURN RECEIPT

NON-CONFIDENTIAL INFORMATION

November 12, 1997

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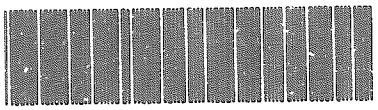

8EHQ-96-13645

Document Processing Center (7407)
Attention: TSCA 8(e) Coordinator
Office of Pollution Prevention and Toxics
U. S. Environmental Protection Agency
401 M Street S. W.
Washington, DC 20460-0001

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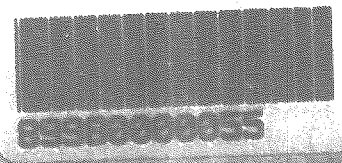
RE: Supplemental TSCA 8(e) Information for Tertiary Amyl Methyl Ether (TAME)

Dear Sir or Madam:

This information is submitted as supplemental information to a previous Section 8(e) notification submitted on behalf of Amerada Hess Corporation, Chevron Products Company, CITGO Petroleum, Exxon Company USA, Marathon Oil Company, Sun Refining and Marketing, and Texaco Refining and Marketing. A copy of our original April 29, 1996 letter is enclosed.

This new information was developed in response to test results obtained under the Enforceable Consent Agreement (ECA, 54 FR 14910 -- March 21, 1995) for TAME (CAS No. 994-05-8). In the initial study that was the subject of the TSCA 8(e) submission, positive findings were observed in an in vitro study of chromosome aberrations in Chinese Hamster Ovary (CHO) cells. We have repeated that study and have used the same protocol to evaluate a metabolite of TAME, tertiary amyl alcohol (CAS No. 75-85-4).

The repeat study with TAME confirmed the results of the first assay. The percentage of cells with aberrations increased in a dose related manner in the S-9 activated test system. The metabolism of TAME (which was studied as part of the ECA) forms tertiary amyl alcohol (TAA) and formaldehyde. The S9 fraction added to the CHO assay will metabolize TAME to those two metabolites. Under the in vitro conditions of this study, we believe that formaldehyde can accumulate and is responsible for the observed effects. This hypothesis is strongly supported by



the experiment with TAA. With TAA as the starting material, no formaldehyde is formed in the test system. As expected, when TAA was tested no increase in chromosome aberrations was seen with or without S9 activation. The hypothesis is also supported by studies with a similar material, methyl tertiary butyl ether (MTBE). MTBE is metabolized to formaldehyde and tertiary butyl alcohol. The effect of MTBE in the in vitro mouse lymphoma assay was demonstrated to be due to the accumulation of formaldehyde (Mackerer et al., Proceedings of the Society of Experimental Biology and Medicine, 1996, Vol. 212, p338-341).

As pointed out above, the toxicity associated with the metabolism of TAME to formaldehyde is likely an artifact of the study conditions and is not expected to occur in vivo. Recent studies with mouse and rat hepatocytes demonstrated that formaldehyde from MTBE metabolism did not accumulate in intact cells (Casanova and Heck, Toxicologist, 1997, Vol. 36, No. 1, p339). Also, formaldehyde is a metabolite from many endogenous substrates and has an essential role in several biosynthetic processes.

Unlike the first study, a statistically significant increase in chromosome aberrations was seen in the non-activated test conditions. However, significantly more cytotoxicity was seen in the non-activated system during the second test and is likely to be the cause of the observation. This interpretation (lack of genotoxic effects) is consistent with the results of other genotoxicity assays with TAME including the Ames test and In Vivo mouse micronucleus studies that were negative (Daughtrey and Bird, Journal of Applied Toxicology, 1995, Vol. 15, Number 4, P313-319). In addition, an HGPRT gene mutation assay in CHO cells was conducted as part of the ECA and was also negative.

If you have any questions about this submission, please contact Ms. CeCe Sharp at the American Petroleum Institute (202) 682-8333.

Sincerely,

Richard D. Cavalli

Richard D. Cavalli
Manager, Toxicology and Health Risk Assessment

Enclosures: 3
1) TSCA 8(e) letter of April 29, 1996
2) Final report: Chromosome Aberrations in Chinese Hamster Ovary (CHO) Cells with Tertiary Amyl Methyl Ether (TAME). Microbiological Associates, Inc. July 31, 1997.
3) Final report: Chromosome Aberrations in Chinese Hamster Ovary (CHO) Cells with Tertiary Amyl Alcohol. Microbiological Associates, Inc. July 31, 1997.

cc. Mr. Gary Timm
Chemical Control Division
Office of Pollution Prevention and Toxics
U. S. Environmental Protection Agency
401 M Street S. W.
Washington, DC 20460

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NON-CONFIDENTIAL INFORMATION

April 29, 1996

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P.O. Box 4054
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Richard B. Cornell
Manager
Toxicology and Health Risk Assessment
Phone 510 242 7911
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TSCA 8(e) Submission for Tertiary Amyl Methyl Ether (TAME)

Document Processing Center
Attention: TSCA 8(e) Coordinator
Office of Pollution Prevention and Toxics
U. S. Environmental Protection Agency
401 M Street S. W.
Washington, DC 20460

Dear Sir or Madam:

This notice is submitted pursuant to Section 8(e) of the Toxic Substances Control Act on behalf of Amerada Hess Corporation, Chevron Products Company, CITGO Petroleum, Exxon Company USA, Marathon Oil Company, Sun Refining and Marketing, and Tenneco Refining and Marketing. This notice is based on test results obtained under a voluntary testing program for Tertiary Amyl Methyl Ether (CAS No. 994-05-8). The required studies are being coordinated by staff from the American Petroleum Institute.

We are advising the EPA of results from an in vitro study of chromosome aberrations in Chinese Hamster Ovary (CHO) cells. In the S9-activated test system there was a positive response with increasing concentrations of TAME. Considering previous Agency guidance for reporting under Section 8(e), we are notifying the EPA of these statistically significant findings.

This study evaluated concentrations of 0, 625, 1200, 2500, and 5000 micrograms of TAME per milliliter of culture medium. Both non-activated and S-9 activated test conditions were evaluated. At [illegible] of TAME, toxicity (cell growth inhibition relative to the solvent control) was approximately 43% in the non-activated system and 72% in the S-9 activated system. The percentage of cells with chromosome increased in a dose related manner in the S-9 activated test system and was statistically significant at concentrations above 625 ug/mL. No dose related increase was observed in the non-activated test system.

Previous studies on TAME did not demonstrate any evidence of genotoxicity (Daughtrey and Bird, Journal of Applied Toxicology, Vol. 15(4), 313-319,1995). The Ames bacterial assay and the mouse

micronucleus assay were both negative. In addition, an HGPRT gene mutation assay in CHO cells has been completed as part of this voluntary testing agreement and was also negative.

A final report for this study will be forwarded to EPA under the conditions of the Enforceable Consent Agreement (54 FR 14910 -- March 21, 1995) Docket Number OPPTS-42059Q - Tertiary Amyl Methyl Ether. If you have any questions about this submission, please contact Dr. Richard Rhodes at the American Petroleum Institute (202) 682-8490.

Very truly yours,

ORIGINAL SIGNED
R. D. CAVALLI

R. D. Cavalli
Manager, Toxicology and Health
Risk Assessment

cc: Mr. Gary Timm
Chemical Control Division
Office of Pollution Prevention and Toxics
U. S. Environmental Protection Agency
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Washington, DC 20460

bcc: THRA Files

RDW
[illegible]

FINAL REPORT

Study Title

CHROMOSOME ABERRATIONS IN CHINESE HAMSTER OVARY (CHO) CELLS

Test Article

Tertiary Amyl Alcohol, CAS 75-85-4

Authors

Ramadevi Gudi, Ph.D.
Elizabeth H. Schadly, B.S.

Study Completion Date

July 31, 1997

Performing Laboratory

Microbiological Associates, Inc.
9630 Medical Center Drive
Rockville, Maryland 20850

Laboratory Study Number

G96CH57.330

Sponsor Project Number

HES3251-HF-08200TEST

Sponsor

American Petroleum Institute
1220 L Street, Northwest
Washington, D.C. 20005

CHROMOSOME ABERRATIONS IN CHINESE HAMSTER OVARY (CHO) CELLS

FINAL REPORT

Sponsor:	**American Petroleum Institute 1220 L Street, Northwest Washington, D.C. 20005**
Authorized Representative:	**Richard A. Rhoden, Ph.D.**
Performing Laboratory:	**Microbiological Associates, Inc. (MA) 9630 Medical Center Drive Rockville, Maryland 20850**
Test Article I.D.:	**Tertiary Amyl Alcohol, CAS 75-85-4**
Test Article Lot Number:	**66H3416, obtained from Sigma Chemical Company**
Test Article Purity:	**99.8%, (provided by Sigma Chemical Company)**
Sponsor Project Number:	**HES3251-HF-08200TEST**
MA Study No.:	**G96CH57.330**
Test Article Description:	**clear, colorless liquid**
Storage Conditions:	**room temperature, protected from exposure to light**
Test Article Receipt:	**December 6, 1996**
Study Initiation:	**January 15, 1997**
Laboratory Supervisor:	**Elizabeth H. Schadly, B.S.**

Study Director: Ramadevi Gudi July 31, 1997
Ramadevi Gudi, Ph.D. Date

QUALITY ASSURANCE STATEMENT

Study Title: CHROMOSOME ABERRATIONS IN CHINESE HAMSTER OVARY (CHO) CELLS

Study Number: G96CH57.330

Study Director: Ramadevi Gudi, Ph.D.

This study has been divided into a series of in-process phases. Using a random sampling approach, Quality Assurance monitors each of these phases over a series of studies. Procedures, documentation, equipment records, etc., are examined in order to assure that the study is performed in accordance with the U.S. FDA Good Laboratory Practice Regulations (21 CFR 58), the U.S. EPA GLPs (40 CFR 792 and 40 CFR 160), the UK GLP Compliance Programme, the Japanese GLP Standard, and the OECD Principles of Good Laboratory Practice and to assure that the study is conducted according to the protocol and relevant Standard Operating Procedures.

The following are the inspection dates, phases inspected, and report dates of QA inspections of this study.

INSPECT ON 16 JAN 97, TO STUDY DIR 16 JAN 97, TO MGMT 16 JAN 97
PHASE: Protocol Review

INSPECT ON 24 FEB 97, TO STUDY DIR 24 FEB 97, TO MGMT 28 FEB 97
PHASE: Preparation of slides for test and control cells

INSPECT ON 12 MAY 97, TO STUDY DIR 12 MAY 97, TO MGMT 13 MAY 97
PHASE: Draft Report

INSPECT ON 31 JUL 97, TO STUDY DIR 31 JUL 97, TO MGMT 31 JUL 97
PHASE: Draft to Final Report

This report describes the methods and procedures used in the study and the reported results accurately reflect the raw data of the study.

Claire L. Courtemanche

Claire L. Courtemanche, B.S.
QUALITY ASSURANCE

July 31, 1997
DATE

STATEMENT OF COMPLIANCE

Study G96CH57.330 was conducted in compliance with the US FDA Good Laboratory Practice Regulations as published in 21 CFR 58, the US EPA GLP Standards 40 CFR 160 and 40 CFR 792, the UK GLP Compliance Programme, the Japanese GLP Standard and the OECD Principles of Good Laboratory Practice in all material aspects with the following exceptions:

The identity, strength, purity and composition or other characteristics to define the test or control article were not determined by the testing facility.

Analyses to determine the uniformity, concentration, or stability of the test or control mixtures were not performed by the testing facility.

The stability of the test or control article under the test conditions was not determined by the testing facility.

Ramadevi Gudi

Ramadevi Gudi, Ph.D.
Study Director

July 31, 1997
Date

SUMMARY

The test article, Tertiary Amyl Alcohol, CAS 75-85-4, was tested both in the absence and in the presence of an Aroclor®-induced S9-activation system in the chromosome aberration assay using Chinese hamster ovary (CHO) cells. The study was conducted in two phases. A preliminary toxicity test was performed to establish the dose range and cell collection times for the chromosome aberration assay. The chromosome aberration assay was used to evaluate the clastogenic potential of the test article.

Dimethyl sulfoxide (DMSO) was determined to be the solvent of choice based on solubility of the test article and compatibility with the target cells. The test article was soluble in DMSO at 500 mg/ml, the maximum concentration tested.

In the preliminary toxicity assay, the maximum dose tested was 5000 µg/mL. The test article was soluble in treatment medium at all concentrations tested. Selection of dose levels for the chromosome aberration assay was based on cell growth inhibition relative to the solvent control. Cell harvest times were determined after evaluating the test article effect on the cell cycle kinetics by measuring the average generation time (AGT). Toxicity, i.e., cell growth inhibition, was 23% and 31% at the 5000 µg/mL dose level in the non-activated and S9-activated test systems, respectively. Based on these findings, the doses chosen for the chromosome aberration assay ranged from 79 to 5000 µg/mL for both the non-activated and the S9-activated systems. No delay in the average generation time was observed at the highest dose tested in the non-activated and S9-activated studies, 5000 µg/mL. In the absence of cell cycle delay in the non-activated and S9-activated portions of the preliminary toxicity assay, the cell harvest times were set at 12 hours for both the non-activated and S9-activated chromosome aberration studies.

In the chromosome aberration assay, the test article was soluble in treatment medium at all concentrations tested. The cells were treated for 12 hours in the non-activated test system and for 4 hours in the S9-activated test system, and were harvested at 12 hours after treatment initiation for both the non-activated and the S9-activated chromosome aberration studies.

Toxicity, i.e., cell growth inhibition, was 42% and 12% at the highest dose level evaluated for structural chromosome aberrations, 5000 μg/mL in the non-activated and S9-activated test systems, respectively. No statistically significant increases in chromosome aberrations were observed in either the non-activated or S9-activated test systems relative to the solvent control group ($p>0.05$, Fisher's exact test). Based on the findings of this study, Tertiary Amyl Alcohol, CAS 75-85-4 was concluded to be negative for the induction of structural chromosome aberrations in Chinese hamster ovary (CHO) cells.

PURPOSE

The purpose of this study was to evaluate the clastogenic potential of a test article based upon its ability to induce chromosome aberrations in Chinese hamster ovary (CHO) cells.

CHARACTERIZATION OF TEST AND CONTROL ARTICLES

The test article, Tertiary Amyl Alcohol, CAS 75-85-4, was received by Microbiological Associates, Inc. on December 6, 1996 and was assigned the code number 96CH57. The test article was characterized by the supplier as a colorless liquid that should be stored in a cool, dry place. No expiration date was provided. Upon receipt, the test article was described as a clear, colorless liquid and was stored at room temperature, protected from exposure to light. The solvent used to deliver Tertiary Amyl Alcohol, CAS 75-85-4 to the test system was DMSO (CAS No.: 67-68-5), obtained from the Fisher Scientific Company.

Mitomycin C (MMC; CAS No: 50-07-7), was obtained from the Sigma Chemical Company, and was dissolved and diluted in sterile distilled water to stock concentrations of 8 and 15 µg/mL for use as the positive control in the non-activated test system. Cyclophosphamide (CP; CAS No.: 6055-19-2), was obtained from Sigma Chemical Company, and was dissolved and diluted in sterile distilled water to stock concentrations of 1000 and 2000 µg/mL for use as the positive control in the S9-activated test system. For each positive control one dose with sufficient scorable metaphase cells was selected for analysis. The solvent for the test article was used as the solvent control at the same concentration as that found in the test article-treated groups. Complete medium or S9 reaction mixture was used in the untreated control.

MATERIALS AND METHODS

Test System

Chinese hamster ovary (CHO-K_1) cells (repository number CCL 61) were obtained from American Type Culture Collection, Rockville, MD. In order to assure the karyotypic stability of the cell line, working cell stocks were not used beyond passage 20. The freeze lot of cells was

tested using the Hoechst staining procedure and found to be free of mycoplasma contamination. The use of CHO cells has been demonstrated to be an effective method of detection of chemical clastogens (Preston et al., 1981).

Metabolic Activation System

Aroclor 1254-induced rat liver S9 was used as the metabolic activation system. The S9 was prepared from male Sprague-Dawley rats induced with a single intraperitoneal injection of Aroclor 1254, 500 mg/kg, five days prior to sacrifice. The S9 was batch prepared and stored at ≤-70°C until used. Each bulk preparation of S9 was assayed for sterility and its ability to metabolize 2-aminoanthracene and 7,12-dimethylbenz(α)anthracene to forms mutagenic to *Salmonella typhimurium* TA100.

Immediately prior to use, the S9 was thawed and mixed with a cofactor pool to contain 2 mM magnesium chloride, 6 mM potassium chloride, 1 mM glucose-6-phosphate, 1 mM nicotinamide adenine dinucleotide phosphate (NADP) and 20 µl S9 per milliliter medium (McCoy's 5A serum-free medium supplemented with 100 units penicillin and 100 µg streptomycin/ml, and 2 mM L-glutamine).

Solubility Test

A solubility test was conducted to select the solvent. The test was conducted using one or more of the following solvents in the order of preference as listed: purified water, dimethylsulfoxide (DMSO), ethanol, and acetone. The test article was tested to determine the solvent, selected in order of preference, that permitted preparation of the highest soluble or workable stock concentration, up to 500 mg/ml.

Preliminary Toxicity Assay

The preliminary toxicity assay was performed for the purpose of selecting dose levels and harvest times for the chromosome aberration assay and consisted of an evaluation of test article effect on cell growth and cell cycle kinetics. CHO cells were seeded for each treatment condition at approximately 5 x 10^5 cells/25 cm^2 flask and were incubated at 37±1°C in a humidified

atmosphere of 5±1% CO_2 in air for 16-24 hours. Treatment was carried out by refeeding the flasks with 5 ml complete medium (McCoy's 5A medium supplemented with 10% fetal bovine serum (FBS), 100 units penicillin and 100 µg streptomycin/ml, and 2 mM L-glutamine) for the non-activated study or S9-reaction mixture (4 ml complete medium plus 1 ml of 5X S9 mix) for the activated study, to which was added 50 µl dosing solution of test article in solvent or solvent alone. The cells were treated for 6 hours without S9 or for 4 hours with S9. Two hours after initiation of treatment, a 50 µl aliquot of 1 mM 5-bromo-2'-deoxyuridine (BrdU) was added to each flask and incubation continued as required. At completion of the exposure period, the treatment medium was removed, the cells washed with calcium and magnesium-free phosphate buffered saline (CMF-PBS), refed with 5 ml complete medium containing 0.01 mM BrdU and returned to the incubator for a total of 24 hours from the initiation of BrdU treatment. Two hours prior to cell harvest, Colcemid® was added to each flask at a final concentration of 0.1 µg/mL. After incubation in Colcemid®, the cells were harvested by trypsinization and counted using a Coulter counter. Cell viability was determined by trypan blue dye exclusion. The cell counts and percent viability were used to determine cell growth inhibition relative to the solvent control. Metaphase preparations were prepared and stained for sister chromatid differentiation using a modified fluorescence-plus-Giemsa technique (Perry and Wolff, 1974). Slides were evaluated for the percentage of first, second and third-plus-subsequent-division metaphase cells for estimation of the test article effect on cell cycle kinetics. The average generation time (AGT) was calculated for each treatment condition.

Chromosome Aberration Assay

The chromosome aberration assay was performed using standard procedures (Evans, 1976), by exposing duplicate cultures of CHO cells to the test article as well as positive, solvent and untreated controls. For the chromosome aberration assay, CHO cells were seeded at approximately 5 x 10^5 cells/25 cm^2 flask and were incubated at 37±1°C in a humidified atmosphere of 5±1% CO_2 in air for 16-24 hours. Treatment was carried out by refeeding duplicate flasks with 5 ml complete medium (McCoy's 5A medium supplemented with 10% FBS, 100 units penicillin and 100 µg streptomycin/mL, and 2 mM L-glutamine) for the non-activated study or 5 ml S9 reaction mixture for the S9-activated study, to which was added 50 µl of dosing

solution of test or control article in solvent or solvent alone. An untreated control consisting of cells in complete medium or S9 reaction mixture was also included.

In the non-activated study, the cells were exposed to the test article continuously up to cell harvest at 37±1°C in a humidified atmosphere of 5±1% CO_2 in air. Two hours prior to the scheduled cell harvest, Colcemid® was added to duplicate flasks for each treatment condition at a final concentration of 0.1 µg/mL and the flasks returned to the incubator until cell collection.

In the S9-activated study, the cells were exposed for 4 hours at 37±1°C in a humidified atmosphere of 5±1% CO_2 in air. After the exposure period, the treatment medium was removed, the cells washed with CMF-PBS, refed with complete medium and returned to the incubator. Two hours prior to the scheduled cell harvest, Colcemid® was added to duplicate flasks for each treatment condition at a final concentration of 0.1 µg/mL and the flasks were returned to the incubator until cell collection.

A concurrent toxicity test was conducted in both the non-activated and the S9-activated studies. After cell harvest an aliquot of the cell suspension was removed from each culture and counted using a Coulter counter. Cell viability was determined by trypan blue dye exclusion. The cell counts and percent viability were used to determine cell growth inhibition relative to the solvent control.

Collection of Metaphase Cells

Two hours after the addition of Colcemid®, metaphase cells were harvested for both the non-activated and S9-activated studies by trypsinization. Cells were collected approximately 12 hours after initiation of treatment. The cells were collected by centrifugation at approximately 800 rpm for 5 minutes. The cell pellet was resuspended in 2-4 ml 0.075 M potassium chloride (KCl) allowed to stand at room temperature for 4-8 minutes. The cells were collected by centrifugation, the supernatant aspirated and the cells fixed with two washes of approximately 2 ml Carnoy's fixative (methanol:glacial acetic acid, 3:1, v/v). The cells were stored overnight or longer in fixative at approximately 2-6°C.

Slide Preparation

To prepare slides, the fixed cells were centrifuged at approximately 800 rpm for 5 minutes, the supernatant was aspirated, and 1 ml fresh fixative was added. After additional centrifugation (at approximately 800 rpm for 5 minutes) the supernatant fluid was decanted and the cells resuspended to opalescence in fresh fixative. A sufficient amount of cell suspension was dropped onto the center of a glass slide and allowed to air dry overnight. Slides were identified by the study number, date prepared and the treatment condition. The dried slides were stained with 5% Giemsa, air dried and permanently mounted.

Evaluation of Metaphase Cells

Slides were coded using random numbers by an individual not involved with the scoring process. To ensure that a sufficient number of metaphase cells were present on the slides, the percentage of cells in mitosis per 500 cells scored (mitotic index) was determined for each treatment group. Metaphase cells with 20±2 centromeres were examined under oil immersion without prior knowledge of treatment groups. Whenever possible, a minimum of 200 metaphase spreads (100 per duplicate flask) were examined and scored for chromatid-type and chromosome-type aberrations. Chromatid-type aberrations include chromatid and isochromatid breaks and exchange figures such as quadriradials (symmetrical and asymmetrical interchanges), triradials, and complex rearrangements. Chromosome-type aberrations include chromosome breaks and exchange figures such as dicentrics and rings. Fragments (chromatid or acentric) observed in the absence of any exchange figure were scored as a break (chromatid or chromosome). Fragments observed with an exchange figure were not scored as an aberration but instead were considered part of the incomplete exchange. Pulverized chromosome(s), pulverized cells and severely damaged cells (≥10 aberrations) were also recorded. Chromatid and isochromatid gaps were recorded but not included in the analysis. The XY coordinates for each cell with chromosomal aberrations were recorded using a calibrated microscope stage.

Evaluation of Test Results

The toxic effects of treatment were based upon cell growth inhibition relative to the solvent-treated control and are presented for the toxicity and aberration studies. The average

generation time (AGT), an estimate of the time for one cell cycle, was calculated for each treatment condition in the preliminary toxicity study as: AGT = (24 hours x 100)/ [(number M_1 cells x 1)+(number M_2 cells x 2)+(number M_3 cells x 3)]. The AGT was used to adjust the cell harvest time in the chromosome aberration assay.

The number and types of aberrations found, the percentage of structurally damaged cells (percent aberrant cells) in the total population of cells examined, and the frequency of structural aberrations per cell (mean aberrations per cell) were calculated and reported for each group. Chromatid and isochromatid gaps are presented in the data but are not included in the total percentage of cells with one or more aberrations or in the frequency of structural aberrations per cell.

Statistical analysis of the percent aberrant cells was performed using the Fisher's exact test. Fisher's test was used to compare pairwise the percent aberrant cells of each treatment group with that of the solvent control. In the event of a positive Fisher's test at any test article dose level, the Cochran-Armitage test was used to measure dose-responsiveness.

All conclusions were based on sound scientific basis; however, as a guide to interpretation of the data, the test article was considered to induce a positive response when the percentages of cells with aberrations were increased in a dose-responsive manner with one or more concentrations being statistically elevated relative to the solvent control group ($p \leq 0.05$). A significant increase at the high dose only with no dose response was considered suspect. A significant increase at one dose level other than the high dose with no dose response was considered equivocal. Test articles that did not demonstrate a statistically significant increase in aberrations were concluded to be negative.

Criteria for a Valid Test

The frequency of cells with structural chromosome aberrations in either the untreated or solvent control must be no greater than 6%. The percentage of cells with chromosome

aberrations in the positive control must be statistically increased ($p \leq 0.05$, Fisher's exact test) relative to the solvent control or to the untreated control if a solvent other than water was used.

Archives

Upon completion of the final report, all raw data, reports, and stained and coded slides are maintained in the archives of Microbiological Associates, Inc., located in Rockville, Maryland.

RESULTS AND DISCUSSION

Solubility Test

DMSO was determined to be the solvent of choice based on solubility of the test article and compatibility with the target cells. The test article was soluble in DMSO at a concentration of 500 mg/ml, the maximum concentration tested.

Preliminary Toxicity Assay

Dose levels and post-treatment cell harvest times for the chromosome aberration assay were selected following a preliminary toxicity test based upon a reduction of cell growth (cell growth inhibition) and cell cycle delay after treatment relative to the solvent control. The results of the evaluation of cell growth inhibition are presented in Tables 1 and 2 and the results of the evaluation of cell cycle delay are presented in Tables 3 and 4. CHO cells were exposed to solvent alone and to nine concentrations of test article ranging from 0.5 µg/mL to 5000 µg/mL in the absence and presence of an S9 reaction mixture. The test article was soluble in treatment medium at all concentrations tested. The osmolality in treatment medium of the highest concentration tested, 5000 µg/mL, was 342 mmol/kg. The osmolality of the solvent in treatment medium was 426 mmol/kg. The pH of the highest concentration of test article in treatment medium was approximately 7.0. Cell growth inhibition relative to the solvent control was 23% and 31% at 5000 µg/mL, the highest concentration tested in the non-activated and S9-activated test systems, respectively. No delay in the average generation time was observed at the highest dose tested in the non-activated or S9-activated studies, 5000 µg/mL. In the absence of cell cycle delay in the preliminary toxicity assay, the cell harvest times for the chromosome aberration assay

were set at 12 hours for both the non-activated and S9-activated chromosome aberration studies, to ensure microscopic evaluation of first-division metaphase cells. Based upon the results of the toxicity study, the dose levels selected for testing in the chromosome aberration assay were 79, 157, 313, 625, 1250, 2500, and 5000 µg/mL.

Chromosome Aberration Assay

The activity of Tertiary Amyl Alcohol, CAS 75-85-4 in the induction of chromosome aberrations in CHO cells when treated in the absence of S9-activation is presented by treatment flask in Table 6 and summarized by group in Table 9. The test article was soluble in treatment medium at all concentrations tested. Toxicity (cell growth inhibition relative to the solvent control) was approximately 42% at 5000 µg/mL, the highest test concentration evaluated for structural chromosome aberrations (Table 5). The mitotic index of the highest dose level evlauated for chromosome aberrations, 5000 µg/mL, was 9% reduced relative to the solvent control. Concentrations of 625, 1250, 2500, and 5000 µg/ml were evaluated for chromosome aberrations. Additional concentrations of 79, 157, and 313 µg/mL were tested as a safeguard against excessive toxicity at higher concentrations but were not required for microscopic examination. The percentage of cells with structural aberrations in the test article-treated groups was not significantly increased above that of the solvent control ($p>0.05$, Fisher's exact test). The percentage of damaged cells in the MMC group was 8% ($p\leq0.01$, Fisher's exact test).

The activity of Tertiary Amyl Alcohol, CAS 75-85-4 in the induction of chromosome aberrations in CHO cells when treated in the presence of an S9 reaction mixture is presented by treatment flask in Table 8 and summarized by group in Table 9. The test article was soluble in treatment medium at all concentrations tested. Toxicity (cell growth inhibition relative to the solvent control) was approximately 12% at 5000 µg/mL, the highest test concentration evaluated for structural chromosome aberrations (Table 7). The mitotic index of the highest dose level evlauated for chromosome aberrations, 5000 µg/mL, was 29% reduced relative to the solvent control. Concentrations of 625, 1250, 2500, and 5000 µg/ml were evaluated for chromosome aberrations. Additional concentrations of 79, 157, and 313 µg/mL were tested as a safeguard against excessive toxicity at higher concentrations but were not required for microscopic

examination. The percentage of cells with structural aberrations in the test article-treated groups was not statistically increased above that of the solvent control ($p>0.05$, Fisher's exact test). The percentage of damaged cells in the CP group was 24% ($p\leq0.01$, Fisher's exact test).

CONCLUSION

The positive and negative controls fulfilled the requirements for a valid test.

Under the conditions of the assay described in this report, Tertiary Amyl Alcohol, CAS 75-85-4 was concluded to be negative in the chromosome aberration assay using Chinese hamster ovary (CHO) cells.

REFERENCES

Evans, H.J. (1976) Cytological methods for detecting chemical mutagens, in: A. Hollaender (Ed.), *Chemical Mutagens, Principles and Methods for their Detection*, vol 4. Plenum Press, New York.

Perry, P., and S. Wolff (1974) New Giemsa method for differential staining of sister chromatids, *Nature*, 251:156-158.

Preston, R.J., W. Au, M.A. Bender, J.G. Brewen, A.V. Carrano, J.A. Heddle, A.F. McFee, S. Wolff and J.S. Wassom (1981) Mammalian *in vivo* and *in vitro* cytogenetic assays: a report of the Gene-Tox Program, *Mutation Research*, 87:143-188.

TABLE 1
PRELIMINARY TOXICITY TEST USING TERTIARY AMYL ALCOHOL, CAS 75-85-4 IN THE ABSENCE OF EXOGENOUS METABOLIC ACTIVATION

6 HOUR TREATMENT, 20 HOUR RECOVERY PERIOD

Treatment[1]	Cell Count ($\times 10^6$)	Cell Viability[2] (%)	Viable Cells per Flask[3] ($\times 10^6$)	Cell Growth Index[4] (%)	Cell Growth Inhibition[5] (%)
DMSO	3.86	92%	3.55	100%	N/C
Tertiary Amyl Alcohol, CAS 75-85-4					
0.5 µg/mL	4.25	90%	3.83	108%	-8%
1.5 µg/mL	3.67	91%	3.34	94%	6%
5 µg/mL	3.93	93%	3.66	103%	-3%
15 µg/mL	3.64	91%	3.32	93%	7%
50 µg/mL	4.19	91%	3.81	107%	-7%
150 µg/mL	3.77	92%	3.47	98%	2%
500 µg/mL	3.02	93%	2.81	79%	21%
1500 µg/mL	3.42	96%	3.29	92%	8%
5000 µg/mL	3.11	88%	2.74	77%	23%

[1] CHO cells were treated in the absence of an exogenous source of metabolic activation for 6 hours at 37±1°C.
[2] Viability determined by trypan blue dye exclusion.
[3] Viable cells/flask = cell count x % viable cells
[4] Growth index = (cells per flask treated group/cells per flask control group), expressed as a percentage.
[5] Cell growth inhibition = 100% - % cell growth index.

TABLE 2
PRELIMINARY TOXICITY TEST USING TERTIARY AMYL ALCOHOL, CAS 75-85-4 IN THE PRESENCE OF EXOGENOUS METABOLIC ACTIVATION

4 HOUR TREATMENT, 22 HOUR RECOVERY PERIOD

Treatment[1]	Cell Count ($x10^5$)	Cell Viability[2] (%)	Viable Cells per Flask[3] ($x10^5$)	Cell Growth Index[4] (%)	Cell Growth Inhibition[5] (%)
DMSO	3.22	93%	3.00	100%	N/C
Tertiary Amyl Alcohol, CAS 75-85-4					
0.5 µg/mL	2.71	87%	2.36	79%	21%
1.5 µg/mL	2.84	88%	2.50	83%	17%
5 µg/mL	2.40	90%	2.16	72%	28%
15 µg/mL	3.14	96%	3.02	101%	-1%
50 µg/mL	2.67	89%	2.38	79%	21%
150 µg/mL	2.63	82%	2.16	72%	28%
500 µg/mL	2.78	89%	2.48	83%	17%
1500 µg/mL	2.47	93%	2.30	77%	23%
5000 µg/mL	2.54	82%	2.08	69%	31%

[1] CHO cells were treated in the presence of an exogenous source of metabolic activation for 4 hours at 37±1°C.
[2] Viability determined by trypan blue dye exclusion.
[3] Viable cells/flask = cell count x % viable cells
[4] Growth index = (cells per flask treated group/cells per flask control group), expressed as a percentage.
[5] Cell growth inhibition = 100% - % cell growth index.

TABLE 3

PRELIMINARY TOXICITY TEST USING TERTIARY AMYL ALCOHOL, CAS 75-85-4 IN THE ABSENCE OF EXOGENOUS METABOLIC ACTIVATION

Treatment[1]	Cell Cycle Kinetics Percentage of cells in M_1	M_2	M_3	Average Generation Time[2] (AGT)
DMSO	1	99	0	12.1
Tertiary Amyl Alcohol, CAS 75-85-4				
0.5 µg/mL	0	100	0	12.0
1.5 µg/mL	0	100	0	12.0
5 µg/mL	0	100	0	12.0
15 µg/mL	0	100	0	12.0
50 µg/mL	0	100	0	12.0
150 µg/mL	0	100	0	12.0
500 µg/mL	2	98	0	12.1
1500 µg/mL	0	100	0	12.0
5000 µg/mL	6	94	0	12.4

[1] CHO cells were treated in the absence of an exogenous source of metabolic activation for 6 hours at 37±1°C. Metaphase cells were collected following a 24 hour growth period in BrdU.

[2] Average Generation Time:

$$\frac{24 \text{ hours} \times 100}{[(\text{number of M1 cells} \times 1) + (\text{number of M2 cells} \times 2) + (\text{number of M3 cells} \times 3)]}$$

TABLE 4

PRELIMINARY TOXICITY TEST USING TERTIARY AMYL ALCOHOL, CAS 75-85-4 IN THE PRESENCE OF EXOGENOUS METABOLIC ACTIVATION

Treatment[1]	Cell Cycle Kinetics Percentage of cells in M_1	M_2	M_3	Average Generation Time[2] (AGT)
DMSO	9	91	0	12.6
Tertiary Amyl Alcohol, CAS 75-85-4				
0.5 µg/mL	3	97	0	12.2
1.5 ug.ml	2	98	0	12.1
5 µg/mL	3	97	0	12.2
15 µg/mL	0	100	0	12.0
50 µg/mL	1	99	0	12.1
150 µg/mL	1	99	0	12.1
500 µg/mL	2	98	0	12.1
1500 µg/mL	1	99	0	12.1
5000 µg/mL	2	98	0	12.1

[1] CHO cells were treated in the presence of an exogenous source of metabolic activation for 4 hours at 37±1°C. Metaphase cells were collected following a 24 hour growth period in BrdU.

[2] Average Generation Time:

$$\frac{24 \text{ hours} \times 100}{[(\text{number of M1 cells} \times 1) + (\text{number of M2 cells} \times 2) + (\text{number of M3 cells} \times 3)]}$$

TABLE 5

CONCURRENT TOXICITY TEST USING TERTIARY AMYL ALCOHOL, CAS 75-85-4 IN THE ABSENCE OF EXOGENOUS METABOLIC ACTIVATION

12 HOUR CONTINUOUS TREATMENT

Treatment[1]	Replicate Flask	Cell Count ($\times 10^5$)	Cell Viability[2] (%)	Mean Viable Cells per Flask[3] ($\times 10^5$)	Cell Growth Index[4] (%)	Cell Growth Inhibition[5] (%)
Untreated	A	1.51	97%			
	B	1.71	91%	1.51	N/C	N/C
DMSO	A	1.73	92%			
	B	1.60	95%	1.55	100%	0%
Tertiary Amyl Alcohol, CAS 75-85-4						
79 µg/mL	A	1.50	93%			
	B	1.81	92%	1.53	99%	1%
157 µg/mL	A	1.56	91%			
	B	1.88	88%	1.54	99%	1%
313 µg/mL	A	1.37	96%			
	B	1.56	89%	1.35	87%	13%
625 µg/mL	A	1.43	90%			
	B	1.53	90%	1.33	86%	14%
1250 µg/mL	A	1.55	94%			
	B	1.14	91%	1.25	80%	20%
2500 µg/mL	A	1.35	92%			
	B	1.30	94%	1.23	79%	21%
5000 µg/mL	A	0.83	93%			
	B	1.06	97%	0.90	58%	42%
MMC,	A	1.32	90%			
0.08 µg/mL	B	1.28	84%	1.13	75%	25%
MMC	A	1.28	83%			
0.15 µg/mL	B	1.29	87%	1.09	72%	28%

[1] CHO cells were treated in the absence of an exogenous source of metabolic activation for 12 hours at 37±1°C.
[2] Viability determined by trypan blue dye exclusion.
[3] Viable cells/flask = cell count x % viable cells, reported as mean of Flasks A and B.
[4] Growth index = (mean cells per flask treated group/mean cells per flask control group), expressed as a percentage. (Test article concentrations compared to solvent control and positive control compared to untreated control.)
[5] Cell growth inhibition = 100% - % cell growth index.

TABLE 6

CYTOGENETIC ANALYSIS OF CHO CELLS TREATED WITH TERTIARY AMYL ALCOHOL, CAS 75-85-4 IN THE ABSENCE OF EXOGENOUS METABOLIC ACTIVATION

12 HOUR CONTINUOUS TREATMENT

Treatment[1,7]	Flask	Mitotic Index	Cells Scored	Aberrant Cells[2] (%)	Total Number of Structural Aberrations: Chromatid-type[3]: Gaps	Breaks	Exch	Chromosome-type[4]: Breaks	Dic	Ring	Severely Damaged Cells[5]	Average Aberrations Per Cell[6]
Untreated cells	A	14.0	100	0	0	0	0	0	0	0	0	0.000
	B	15.0	100	0	0	0	0	0	0	0	0	0.000
DMSO	A	14.8	100	2	1	0	0	1	0	1	0	0.020
	B	16.8	100	2	0	1	0	1	0	0	0	0.020
Tertiary Amyl Alcohol, CAS 75-85-4												
625 µg/mL	A	11.4	100	0	1	0	0	0	0	0	0	0.000
	B	13.0	100	1	0	1	0	0	0	0	0	0.010
1250 µg/mL	A	16.0	100	0	1	0	0	0	0	0	0	0.000
	B	14.6	100	0	1	0	0	0	0	0	0	0.000
2500 µg/mL	A	18.8	100	3	0	1	0	2	0	0	0	0.030
	B	15.2	100	1	0	0	0	1	0	0	0	0.010
5000 µg/mL	A	14.8	100	0	0	0	0	0	0	0	0	0.000
	B	13.8	100	1	0	1	0	0	0	0	0	0.010
MMC	A	8.4	100	9	2	3	3	3	0	0	0	0.090
0.15 µg/mL	B	6.2	100	7	3	3	2	2	0	0	0	0.070

1 CHO cells were treated for 12 hours at 37±1°C in the absence of an exogenous source of metabolic activation.
2 Excluding cells with only gaps.
3 Chromatid breaks include chromatid and isochromatid breaks and fragments; chromatid exchange figures include quadriradials, triradials and complex rearrangements.
4 Chromosome breaks include breaks and acentric fragments; dic, dicentric chromosome.
5 Severely damaged cells include cells with one or more pulverized chromosomes and cells with 10 or more aberrations.
6 Severely damaged cells and pulverizations were counted as 10 aberrations.
7 Additional dose levels of 79, 157, and 313 µg/mL were tested as a guard against excessive toxicity at higher dose levels but were not required for microscopic examination.

TABLE 7

CONCURRENT TOXICITY TEST USING TERTIARY AMYL ALCOHOL, CAS 75-85-4 IN THE PRESENCE OF EXOGENOUS METABOLIC ACTIVATION

4 HOUR TREATMENT, 8 HOUR RECOVERY PERIOD

Treatment[1]	Replicate Flask	Cell Count ($\times 10^5$)	Cell Viability[2] (%)	Mean Viable Cells per Flask[3] ($\times 10^5$)	Cell Growth Index[4] (%)	Cell Growth Inhibition[5] (%)
Untreated	A	1.41	95%			
	B	1.33	97%	1.31	N/C	N/C
DMSO	A	1.44	95%			
	B	1.23	98%	1.29	100%	0%
Tertiary Amyl Alcohol, CAS 75-85-4						
79 µg/mL	A	1.58	99%			
	B	1.25	94%	1.37	107%	-7%
157 µg/mL	A	1.52	96%			
	B	1.19	92%	1.28	99%	1%
313 µg/mL	A	1.37	96%			
	B	1.28	95%	1.27	99%	1%
625 µg/mL	A	1.09	98%			
	B	1.36	100%	1.21	94%	6%
1250 µg/mL	A	1.17	97%			
	B	1.22	98%	1.17	91%	9%
2500 µg/mL	A	1.04	93%			
	B	1.26	99%	1.11	86%	14%
5000 µg/mL	A	1.15	97%			
	B	1.21	96%	1.14	88%	12%
CP, 10 µg/mL	A	1.56	95%			
	B	1.31	92%	1.34	102%	-2%
CP, 20 µg/mL	A	1.18	89%			
	B	1.35	92%	1.15	87%	13%

[1] CHO cells were treated in the presence of an exogenous source of metabolic activation for 4 hours at 37±1°C.
[2] Viability determined by trypan blue dye exclusion.
[3] Viable cells/flask = cell count x % viable cells, reported as mean of Flasks A and B.
[4] Growth index = (mean cells per flask treated group/mean cells per flask control group), expressed as a percentage. (Test article concentrations compared to solvent control and positive control compared to untreated control.)
[5] Cell growth inhibition = 100% - % cell growth index.

TABLE 8

CYTOGENETIC ANALYSIS OF CHO CELLS TREATED WITH Tertiary Amyl Alcohol, CAS 75-85-4 IN THE PRESENCE OF EXOGENOUS METABOLIC ACTIVATION

4 HOUR TREATMENT, 8 HOUR RECOVERY PERIOD

Treatment[1,7]	Flask	Mitotic Index	Cells Scored	Aberrant Cells[2] (%)	Total Number of Structural Aberrations: Chromatid-type[3] Gaps	Chromatid-type[3] Breaks	Chromatid-type[3] Exch	Chromosome-type[4] Breaks	Chromosome-type[4] Dic	Chromosome-type[4] Ring	Severely Damaged Cells[5]	Average Aberrations Per Cell[6,8]
Untreated cells	A	25.4	100	0	2	0	0	0	0	0	0	0.000
	B	26.0	100	0	1	0	0	0	0	0	0	0.000
DMSO	A	26.2	100	4	2	4	0	0	1	0	0	0.050
	B	25.6	100	1	1	1	0	0	0	0	0	0.010
Tertiary Amyl Alcohol, CAS 75-85-4												
625 µg/mL	A	16.6	100	2	0	1	1	0	0	0	0	0.020
	B	19.0	100	1	1	0	0	0	0	1	0	0.010
1250 µg/mL	A	20.4	100	4	1	4	0	0	0	0	0	0.040
	B	21.0	100	3	0	2	0	0	0	1	0	0.030
2500 µg/mL	A	23.2	100	3	1	1	0	1	0	1	0	0.030
	B	22.2	100	3	2	1	1	0	0	1	0	0.030
5000 µg/mL	A	19.2	100	4	1	5	0	0	1	0	0	0.060
	B	17.4	100	1	1	0	0	1	0	0	0	0.010
CP	A	8.4	100	27	0	18	5	8	0	0	1	0.410
10 µg/mL	B	10.4	100	21	1	22	3	4	1	0	0	0.380

1 CHO cells were treated for 4 hours at 37±1°C in the presence of an exogenous source of metabolic activation.
2 Excluding cells with only gaps.
3 Chromatid breaks include chromatid and isochromatid breaks and fragments; chromatid exchange figures include quadriradials, triradials and complex rearrangements.
4 Chromosome breaks include breaks and acentric fragments; dic, dicentric chromosome.
5 Severely damaged cells include cells with one or more pulverized chromosomes and cells with 10 or more aberrations.
6 Severely damaged cells and pulverizations were counted as 10 aberrations.
7 Additional dose levels of 79, 157, and 313 µg/mL were tested as a guard against excessive toxicity at higher dose levels but were not required for microscopic examination.

TABLE 9

SUMMARY

Treatment	S9 Activation	Treatment/Harvest Time (hrs)	Mitotic Index	Cells Scored	Aberrations Per Cell[1] (Mean ± SD)	Cells With Aberrations[2] (%)
Untreated	-	12/12	14.5	200	0.000 ± 0.000	0.0
DMSO	-	12/12	15.8	200	0.020 ± 0.140	2.0
Tertiary Amyl Alcohol, CAS 75-85-4						
625 µg/mL	-	12/12	12.2	200	0.005 ± 0.071	0.5
1250 µg/mL	-	12/12	15.3	200	0.000 ± 0.000	0.0
2500 µg/mL	-	12/12	17.0	200	0.020 ± 0.140	2.0
5000 µg/mL	-	12/12	14.3	200	0.005 ± 0.071	0.5
MMC 0.15 µg/mL	-	12/12	7.3	200	0.080 ± 0.272	8.0**
Untreated	+	4/12	25.7	200	0.000 ± 0.000	0.0
DMSO	+	4/12	25.9	200	0.030 ± 0.198	2.5
Tertiary Amyl Alcohol, CAS 75-85-4						
625 µg/mL	+	4/12	17.8	200	0.015 ± 0.122	1.5
1250 µg/mL	+	4/12	20.7	200	0.035 ± 0.184	3.5
2500 µg/mL	+	4/12	22.7	200	0.030 ± 0.171	3.0
5000 µg/mL	+	4/12	18.3	200	0.035 ± 0.232	2.5
CP 10 µg/mL	+	4/12	9.4	200	0.355 ± 0.913	24.0**

[1] Severely damaged cells were counted as 10 aberrations.
[2] *, $p \leq 0.05$, **, $p \leq 0.01$; Fisher's exact test.

APPENDIX I

Historical Control Data

IN VITRO MAMMALIAN CYTOGENETIC TEST USING CHINESE HAMSTER OVARY (CHO) CELLS

HISTORICAL CONTROL VALUES STRUCTURAL ABERRATIONS 1994-1996

NON-ACTIVATED TEST SYSTEM

Historical Values	Aberrant Cells		
	Untreated Control	Solvent Control[1]	Positive Control[2]
Mean	1.0%	1.1%	27.3%
Standard Deviation	1.0%	1.1%	16.4%
Range	0.0% to 5.5%	0.0% to 5.5%	7.5% to 94.0%

S9-ACTIVATED TEST SYSTEM

Historical Values	Aberrant Cells		
	Untreated Control	Solvent Control[1]	Positive Control[3]
Mean	1.4%	1.3%	42.7%
Standard Deviation	1.3%	1.2%	24.6%
Range	0.0% to 5.5%	0.0% to 6.0%	7.5% to 100.0%

[1]Solvents include water, saline, dimethylsulfoxide, ethanol, acetone, non-standard solvents and Sponsor-supplied vehicles.
[2]Positive control for non-activated studies, triethylenemelamine (TEM, 0.25-0.5 µg/mL,) N-methyl-N'-nitro-N-nitrosoguanidine (MNNG, 0.75-2 µg/mL), and Mitomycin C (MMC, 0.08-0.15 µg/mL).
[3]Positive control for S9-activated studies, cyclophosphamide (CP, 10-50 µg/mL), and benzo(α)pyrene, (B[α]P, 30 µg/mL).

APPENDIX II

Study Protocol

APPROVED MA Study Number: G96CH57.330

Chromosome Aberrations in Chinese Hamster Ovary (CHO) Cells

1.0 PURPOSE

The purpose of this study is to evaluate the clastogenic potential of a test article based upon its ability to induce chromosome aberrations in Chinese hamster ovary (CHO) cells.

2.0 SPONSOR

2.1	Name:	American Petroleum Institute
2.2	Address:	1220 L Street, Northwest Washington, D.C. 20005
2.3	Representative:	Richard A. Rhoden, Ph.D.
2.4	Sponsor Project #:	HES3251-HF-08200TEST

3.0 IDENTIFICATION OF TEST AND CONTROL SUBSTANCES

3.1 Test Article: Tertiary Amyl Alcohol, CAS 75-85-4

3.2 Controls:
- Untreated: Untreated Cells
- Solvent: Test Article Solvent
- Positive: Mitomycin C (MMC), Cyclophosphamide (CP)

3.3 Determination of Strength, Purity, etc.

The Sponsor will be directly responsible for determination and documentation of the analytical purity and composition of the test article and the stability and strength of the dosing solutions.

3.4 Test Article Retention Sample

The retention of a reserve sample of the test article will be the responsibility of the Sponsor.

4.0 TESTING FACILITY AND KEY PERSONNEL

4.1	Name:	Toxicology Testing Facility Microbiological Associates, Inc.
4.2	Address:	9630 Medical Center Drive Rockville, MD 20850
4.3	Study Director:	Ramadevi Gudi, Ph.D.

MICROBIOLOGICAL ASSOCIATES, INC.

5.0 TEST SCHEDULE

5.1 Proposed Experimental Initiation Date: 2/17/97

5.2 Proposed Experimental Completion Date: 4/28/97

5.3 Proposed Report Date: 5/12/97

6.0 TEST SYSTEM

The CHO-K_1 cell line is a proline auxotroph with a modal chromosome number of 20 and a population doubling time of 10-14 hours. CHO-K_1 cells were obtained from the American Type Culture Collection (repository number CCL 61), Rockville, MD. This system has been demonstrated to be sensitive to the clastogenic activity of a variety of chemicals (Preston et al., 1981).

7.0 EXPERIMENTAL DESIGN AND METHODOLOGY

The chromosome aberration assay will be conducted using standard procedures (Evans, 1976), by exposing cultures of CHO cells to a minimum of four concentrations of the test article as well as to positive, untreated, and solvent controls. In the non-activated test system, treatment will be continuous up to the time of cell collection; in the S9 activated test system, exposure will be for 4 hours. The dividing cells will be arrested in metaphase and harvested for microscopic evaluation of chromosome aberrations at approximately 12 hours after the initiation of treatment. In order to ensure evaluation of first-division metaphase cells, the cell collection time may be delayed up to 20 hours in the event of cell cycle delay associated with test article treatment. The clastogenic potential of the test article will be measured by its ability to increase structural chromosome aberrations in a dose-responsive manner when compared to the solvent control group.

7.1 Solubility Determination

Unless the Sponsor has indicated the test article solvent, a solubility determination will be conducted to determine the solvent and the maximum soluble concentration up to a maximum of 500 mg/ml. Solvents compatible with this test system, in order of preference, include but are not limited to sterile water (CAS 7732-18-5), dimethylsulfoxide (CAS 67-68-5), ethanol (CAS 64-17-5), and acetone (CAS 67-64-1). The solvent will be the test article solvent, selected in order of preference, that permits preparation of the highest stock concentration, up to 500 mg/ml.

7.2 Dose Levels

Selection of the dose levels for the cytogenetics assay will be based upon cell growth inhibition relative to the solvent control after treatment. CHO cells will be exposed to solvent alone and to at least nine concentrations of test article. The highest concentration tested will be 5 mg/ml for freely soluble test articles, or the maximum concentration resulting in a workable suspension for poorly soluble test articles. The pH will be measured at the highest test article treatment condition.

and will be adjusted, if necessary, in order to maintain a neutral pH in the treatment medium. The osmolality of the highest treatment condition in treatment medium will also be measured. Cells seeded 16-24 hours earlier will be exposed for 6 hours in the absence of S9 and for 4 hours in the presence of S9. Two hours after initiation of exposure, 5-bromo-2'-deoxyuridine (BrdU), will be added to the culture medium at a final concentration of 0.01 mM. The cultures will be grown in medium containing 0.01 mM BrdU for 24 hours. Twenty two hours after BrdU treatment initiation Colcemid® (0.1 µg/ml) will be added to the cultures. Two hours after Colcemid® addition the cells will be harvested by trypsinization and counted using an automatic cell counter and trypan blue dye exclusion. The cell counts and percent viability will be used to determine cell growth inhibition relative to the solvent control. Metaphase preparations will be made and stained for sister chromatid differentiation using a modified fluorescence-plus-Giemsa technique (Perry and Wolff, 1974). Slides will be evaluated for the percentage of first-, second- and third- plus-subsequent-division metaphase cells per 100 cells scored for determination of the test article effect on cell cycle kinetics.

Whenever possible, the high dose for the chromosome aberration assay will be selected to give at least 50% toxicity (cell growth inhibition relative to the solvent). At least three additional dose levels will be included in the chromosome aberration assay. In the event the test article cannot be dissolved at a high enough concentration in an appropriate solvent to be toxic, then the highest dose to be tested will be the concentration resulting in minimum precipitation in test medium. Minimum precipitation will be determined by direct visual inspection. In the event the test article demonstrates a dose-responsive increase in toxicity (cell growth inhibition relative to the solvent) at concentrations that exceed solubility in treatment medium, then the highest dose to be tested will be the maximum concentration resulting in a workable suspension that results in at least 50% toxicity. If excessive precipitation of the test article-solvent solution occurs upon addition to treatment medium, or if the osmolality of the treatment medium is excessive, the Sponsor will be consulted. The cell cycle kinetics study will be used to verify the average cell generation time of the target cells and to measure the test article effect on the cell generation time. Post-treatment cell harvest times will be adjusted if there is considerable cell cycle delay.

7.3 Frequency and Route of Administration

Target cells will be treated for 12 hours in the absence of S9 (or up to 20 hours depending upon cell cycle delay) and for 4 hours in the presence of S9 by incorporation of the test article-solvent mixture into the treatment medium. This technique has demonstrated to be an effective method of detection of chemical clastogens in this test system.

7.4 Activation System

Aroclor 1254-induced rat liver S9 will be used as the metabolic activation system. The S9 will be prepared from male Sprague-Dawley rats induced with a single intraperitoneal injection of Aroclor 1254, 500 mg/kg, five days prior to sacrifice. The S9 will be batch prepared and stored frozen at approximately -70°C until used.

Immediately prior to use, the S9 will be thawed and mixed with cofactors to contain 2 mM magnesium chloride ($MgCl_2$), 6 mM potassium chloride (KCl), 1 mM glucose-6-phosphate, 1 mM nicotinamide adenine dinucleotide phosphate (NADP) and 20 µl S9 per ml serum free medium.

7.5 Controls

7.5.1 Untreated Control

Untreated cells will be used as the untreated control.

7.5.2 Solvent Control

The solvent for the test article will be used as the solvent control. For solvents other than water, physiological buffer or culture medium, the final concentration in treatment medium will not exceed 1%.

7.5.3 Positive Controls

Mitomycin C will be used at a concentration within 0.05-0.3 µg/ml as the positive control in the non-activated study. Cyclophosphamide will be used at a concentration within 10-50 µg/ml as the positive control in the S9-activated study.

7.6 Preparation of Target Cells

Exponentially growing CHO-K_1 cells will be seeded in complete medium (McCoy's 5A medium containing 10% fetal bovine serum, 2 mM L-glutamine, 100 units penicillin/ml and 100 µg streptomycin/ml) for each treatment condition at approximately 5 x 10^5 cells/25 cm^2 flask. The flasks will be incubated at 37 ± 1°C in a humidified atmosphere of 5 ± 1% CO_2 in air for 16-24 hours.

7.7 Identification of Test System

Using a permanent marking pen, the treatment flasks will be identified by the MA study number and a code system to designate the treatment condition and test phase.

7.8 Treatment of Target Cells

Treatment will be carried out in duplicate by refeeding the flasks with 5 ml complete medium for the non-activated exposure or 5 ml S9 reaction mixture for the S9-activated exposure, to which will be added 50 µl of dosing solution of test or control article in solvent or solvent alone. Larger volumes of dosing solution may be used if water, physiological buffer, or medium is used as the solvent. An untreated control consisting of cells in complete medium or S9 reaction mixture will also be included.

In the non-activated study, the cells will be treated for 12 hours at 37 ± 1°C in a humidified atmosphere of 5 ± 1% CO_2 in air. Treatment will be extended to up to 20 hours in those dose levels with considerable cell cycle delay. Treatment will continue until collection of metaphase cells.

In the S9-activated study, the cells will be treated for 4 hours at 37 ± 1°C in a humidified atmosphere of 5 ± 1% CO_2 in air. After the treatment period, the treatment medium will be aspirated, the cells washed with phosphate buffered saline, refed with complete medium and returned to the incubator.

A concurrent toxicity test will be conducted in both the non-activated and the S9-activated studies. After cell harvest an aliquot of the cell suspension will be removed from each culture and counted using an automatic cell counter. Cell viability will be determined by trypan blue dye exclusion. The cell counts and percent viability will be used to determine cell growth inhibition relative to the solvent control.

7.9 Collection of Metaphase Cells

Cells will be collected approximately 12 hours after initiation of treatment or at a later time selected to represent the first division metaphase after initiation of treatment if the test article causes considerable cell cycle delay. Cell fixation will not extend beyond 20 hours after initiation of treatment. Two hours prior to harvest, Colcemid® will be added to the cultures at a final concentration of 0.1 µg/ml.

Cells will be harvested by trypsinization, collected by centrifugation and an aliquot will be removed for counting using an automatic cell counter and trypan blue dye exclusion. The remainder of the cells will be swollen with 0.075M KCl, washed with two consecutive changes of fixative (methanol:glacial acetic acid, 3:1 v/v), capped and stored overnight or longer at approximately 2-6°C. The cell counts and percent viability will be used to determine cell growth inhibition relative to the solvent control. To prepare slides, the cells will be collected by centrifugation and resuspended in fresh fixative. The suspension of fixed cells will be applied dropwise onto a glass microscope slide and air-dried. The slide will be identified by the experiment number, treatment condition and date. The slides will then be stained with Giemsa and permanently mounted.

7.10 Scoring for Metaphase Aberrations

To ensure that a sufficient number of metaphase cells are present on the slides, the percentage of cells in mitosis per 500 cells scored (mitotic index) will be determined for each treatment group. Slides will be coded using random numbers by an individual not involved with the scoring process. Metaphase cells with 20 ± 2 centromeres will be examined under oil immersion without prior knowledge of treatment groups. Whenever possible, a minimum of 200 metaphase spreads from each dose level (100 per duplicate flask) will be examined and scored for chromatid-type and chromosome-type aberrations. Chromatid-type aberrations include chromatid and isochromatid breaks and exchange figures such as quadriradials (symmetrical and asymmetrical interchanges), triradials, and complex rearrangements. Chromosome-type aberrations include chromosome breaks and exchange figures such as dicentrics and rings. Fragments (chromatid or acentric) observed in the absence of any exchange figure will be scored as a break (chromatid or chromosome). Fragments observed with an exchange figure will not be scored as an aberration but will be considered part of the incomplete exchange. Pulverized chromosome(s), pulverized cells and severely damaged cells (≥ 10 aberrations) will also be recorded. Chromatid and isochromatid gaps will be recorded but not included in the analysis. The XY coordinates for each cell with a structural aberration will be recorded using a calibrated microscope stage. The mitotic index will be recorded as the percentage of cells in mitosis per 500 cells counted.

8.0 CRITERIA FOR DETERMINATION OF A VALID TEST

8.1 Untreated and Solvent Controls

The frequency of cells with structural chromosome aberrations in either the untreated or solvent control must be no greater than 6%.

8.2 Positive Control

The percentage of cells with aberrations must be statistically increased ($p \leq 0.05$, Fisher's exact test) relative to the solvent control or to the untreated control if a solvent other than water was used.

9.0 EVALUATION OF TEST RESULTS

The toxic effects of treatment are based upon cell growth inhibition relative to the solvent-treated control and will be presented for the toxicity and aberration studies. The AGT will be calculated for each treatment condition in the toxicity study as: AGT = (24 hours x 100)/[(number M_1 cells x 1)+(number M_2 cells x 2)+(number M_3 cells x 3)]. The number and types of aberrations found, the percentage of structurally damaged cells in the total population of cells examined, and the mean aberrations per cell will be calculated and reported for each treatment group. Chromatid and isochromatid gaps are presented in the data but are not included in the total percentage of cells with one or more aberrations or in the frequency of structural aberrations per cell. Statistical analysis of the

percentage of aberrant cells will be performed using the Fisher's exact test. The Fisher's test will be used to compare pairwise the percent aberrant cells of each treatment group with that of the solvent control. In the event of a positive Fisher's exact test at any test article dose level, the Cochran-Armitage test will be used to measure dose-responsiveness. All conclusions will be based on sound scientific basis; however, as a guide to interpretation of the data, the test article will be considered to induce a positive response when the percentage of cells with aberrations is increased in a dose-responsive manner with one or more concentrations being statistically significant ($p \leq 0.05$). A significant increase at the high dose only with no dose response will be considered suspect. A significant increase at one dose level other than the high dose with no dose response will be considered equivocal. Test articles not demonstrating a statistically significant increase in aberrations will be concluded to be negative.

10.0 REPORT

A report of the results of this study will be prepared by Microbiological Associates, Inc. and will accurately describe all methods used for generation and analysis of the data.

Results presented will include, but not be limited to:

- cells used
- test conditions: composition of medium, CO_2 concentration, incubation time, concentration of test article and rationale for selection of concentration, duration of treatment, duration of treatment with and concentration of Colcemid®, type of metabolic activation system used, positive, untreated, and solvent controls
- number of cell cultures
- mitotic index and number of metaphases analyzed (method for determination; data given separately for each culture)
- cell growth inhibition relative to the solvent control
- criteria for scoring aberrations
- type and number of aberrations, given separately for each treated and control culture
- historical control data

11.0 RECORDS AND ARCHIVES

Upon completion of the final report, all raw data and reports will be maintained by the Quality Assurance Unit of Microbiological Associates, Inc., Rockville, MD, in accordance with the relevant Good Laboratory Practice Regulations.

12.0 REGULATORY REQUIREMENTS/GOOD LABORATORY PRACTICE

This protocol was designed to fulfill OECD Guideline 473 (Genetic Toxicology: *In Vitro* Mammalian Cytogenetic Test), May, 1983; and EPA Health Effects Testing Guidelines, Subpart 796.5375 (*In Vitro* Mammalian Cytogenetics), Fed. Register, vol. 50, September, 1985 with revisions Fed. Register, vol. 52, May, 1987.

This study will be performed in compliance with the provisions of the Good Laboratory Practice Regulations For Non-clinical Laboratory Studies.

Will this study be submitted to a regulatory agency? yes

If so, to which agency or agencies? EPA

Unless arrangements are made to the contrary, unused dosing solutions will be disposed of following administration to the test system and all residual test article will be disposed of following finalization of the report.

13.0 REFERENCES

Evans, H.J. (1976) Cytological methods for detecting chemical mutagens, in: A. Hollaender (Ed.), Chemical Mutagens, Principles and Methods for their Detection, vol. 4. Plenum Press, New York, NY.

Perry, P. and S. Wolff (1974) New Giemsa method for differential staining of sister chromatids, Nature, 251: 156-158.

Preston, R.J., W. Au, M.A. Bender, J.G. Brewen, A.V. Carrano, J.A. Heddle, A.F. McFee, S. Wolff and J.S. Wassom (1981) Mammalian in vivo and in vitro cytogenetic assays: a report of the Gene-Tox Program, Mutation Research, 87:143-188.

14.0 APPROVAL

Richard A. Rhoden 1/8/97
SPONSOR REPRESENTATIVE DATE

Richard A. Rhoden, Ph.D.
(Print or Type Name)

Ramadka Gad 1/16/97
MA STUDY DIRECTOR DATE

FINAL REPORT

Study Title

CHROMOSOME ABERRATIONS IN CHINESE HAMSTER OVARY (CHO) CELLS

Test Article

Tertiary Amyl Methyl Ether (TAME)

Authors

Ramadevi Gudi, Ph.D.
Elizabeth H. Schadly, B.S.

Study Completion Date

July 31, 1997

Performing Laboratory

Microbiological Associates, Inc.
9630 Medical Center Drive
Rockville, Maryland 20850

Laboratory Study Number

G96CJ24.330

Sponsor Project Number

HES3251-HF-08200TEST

Sponsor

American Petroleum Institute
1220 L Street, Northwest
Washington, D.C. 20005

CHROMOSOME ABERRATIONS IN CHINESE HAMSTER OVARY (CHO) CELLS

FINAL REPORT

Sponsor:	**American Petroleum Institute 1220 L Street, Northwest Washington, D.C. 20005**
Authorized Representative:	**Richard A. Rhoden, Ph.D.**
Performing Laboratory:	**Microbiological Associates, Inc. (MA) 9630 Medical Center Drive Rockville, Maryland 20850**
Test Article I.D.:	**Tertiary Amyl Methyl Ether (TAME)**
Test Article Lot Number:	**UN3271**
Test Article Purity:	**98.9%, (provided by Chevron)**
Sponsor Project Number:	**HES3251-HF-08200TEST**
MA Study No.:	**G96CJ24.330**
Test Article Description:	**clear, colorless liquid**
Storage Conditions:	**room temperature, protected from exposure to light**
Test Article Receipt:	**December 16, 1996**
Study Initiation:	**January 15, 1997**
Laboratory Supervisor:	**Elizabeth H. Schadly, B.S.**

Study Director: Ramadevi Gudi July 31, 1997
Ramadevi Gudi, Ph.D. Date

QUALITY ASSURANCE STATEMENT

Study Title: CHROMOSOME ABERRATIONS IN CHINESE HAMSTER OVARY (CHO) CELLS

Study Number: G96CJ24.330

Study Director: Ramadevi Gudi, Ph.D.

This study has been divided into a series of in-process phases. Using a random sampling approach, Quality Assurance monitors each of these phases over a series of studies. Procedures, documentation, equipment records, etc., are examined in order to assure that the study is performed in accordance with the U.S. FDA Good Laboratory Practice Regulations (21 CFR 58), the U.S. EPA GLPs (40 CFR 792 and 40 CFR 160), the UK GLP Compliance Programme, the Japanese GLP Standard, and the OECD Principles of Good Laboratory Practice and to assure that the study is conducted according to the protocol and relevant Standard Operating Procedures.

The following are the inspection dates, phases inspected, and report dates of QA inspections of this study.

INSPECT ON 16 JAN 97, TO STUDY DIR 16 JAN 97, TO MGMT 16 JAN 97
PHASE: Protocol Review

INSPECT ON 25 FEB 97, TO STUDY DIR 25 FEB 97, TO MGMT 28 FEB 97
PHASE: Preparation of S9 mixture

INSPECT ON 12 MAY 97, TO STUDY DIR 12 MAY 97, TO MGMT 13 MAY 97
PHASE: Draft Report

INSPECT ON 31 JUL 97, TO STUDY DIR 31 JUL 97, TO MGMT 04 AUG 97
PHASE: Draft to Final Report

This report describes the methods and procedures used in the study and the reported results accurately reflect the raw data of the study.

Claire L. Courtemanche

Aug 4, 1997

Claire L. Courtemanche, B.S.
QUALITY ASSURANCE

DATE

STATEMENT OF COMPLIANCE

Study G96CJ24.330 was conducted in compliance with the US FDA Good Laboratory Practice Regulations as published in 21 CFR 58, the US EPA GLP Standards 40 CFR 160 and 40 CFR 792, the UK GLP Compliance Programme, the Japanese GLP Standard and the OECD Principles of Good Laboratory Practice in all material aspects with the following exceptions:

The identity, strength, purity and composition or other characteristics to define the test or control article were not determined by the testing facility.

Analyses to determine the uniformity, concentration, or stability of the test or control mixtures were not performed by the testing facility.

The stability of the test or control article under the test conditions was not determined by the testing facility.

Ramadevi Gudi

Ramadevi Gudi, Ph.D.
Study Director

July 31, 1997
Date

SUMMARY

The test article, Tertiary Amyl Methyl Ether (TAME), was tested both in the absence and in the presence of an Aroclor®-induced S9-activation system in the chromosome aberration assay using Chinese hamster ovary (CHO) cells. The chromosome aberration assay was used to evaluate the clastogenic potential of the test article.

Ethanol was determined to be the solvent of choice based on information from the Sponsor, solubility of the test article and compatibility with the target cells. The test article was soluble in ethanol at a maximum concentration of 500 mg/ml and was soluble in the treatment medium at a concentration of 5000 µg/ml.

The dose levels and harvest times for the chromosome aberration assay were selected based upon previous study conducted using the same test article at MA Bioservices. The dose levels ranged from 313 to 5000 µg/mL in both the non-activated and S9-activated test systems. The test article was soluble in treatment medium at all concentrations tested. The cells were treated for 12 hours in the non-activated test system and for 4 hours in the S9-activated test system, and were harvested at 12 hours after treatment initiation for the non-activated study and at 20 hours after treatment initiation for the S9-activated study. Toxicity (cell growth inhibition) was approximately 70% and 48% at the highest dose levels evaluated for chromosome aberrations, 5000 and 2500 µg/mL, in the non-activated and S9-activated studies, respectively. Dose level 5000 µg/mL in the S9-activated assay was not evaluated due to complete mitotic inhibition (no mitotic cells observed). Statistically significant increases in chromosome aberrations were observed in the non-activated test system relative to the solvent control group at dose levels 2500 and 5000 µg/mL ($p \leq 0.01$, Fisher's exact test) and in the S9-activated test system at all dose levels evaluated, 313, 625, 1250, and 2500 µg/mL ($p \leq 0.01$, Fisher's exact test). The positive response seen in the non-activated system only occurred at concentrations of TAME which significantly reduced the mitotic index. A previous study with TAME (Microbiological Associates, Inc., G95BA89.330) did not observe a significant increase in chromosome aberrations at the nominal concentrations. Based on the findings of this study, Tertiary Amyl Methyl Ether (TAME) was

concluded to be positive for the induction of structural chromosome aberrations in Chinese hamster ovary (CHO) cells.

PURPOSE

The purpose of this study was to evaluate the clastogenic potential of a test article based upon its ability to induce chromosome aberrations in Chinese hamster ovary (CHO) cells.

CHARACTERIZATION OF TEST AND CONTROL ARTICLES

The test article, Tertiary Amyl Methyl Ether (TAME), was received by Microbiological Associates, Inc. on December 16, 1996 and was assigned the code number 96CJ24. The test article was characterized by the supplier as a clear liquid that should be stored away from strong oxidizing materials or strong acids. No expiration date was provided. Upon receipt, the test article was described as a clear, colorless liquid and was stored at room temperature, protected from exposure to light. The solvent used to deliver Tertiary Amyl Methyl Ether (TAME) to the test system was ethanol (CAS No.: 64-17-5), obtained from the Fisher Scientific Company.

Mitomycin C (MMC; CAS No: 50-07-7), was obtained from the Sigma Chemical Company, and was dissolved and diluted in sterile distilled water to stock concentrations of 8 and 15 µg/mL for use as the positive control in the non-activated test system. Cyclophosphamide (CP; CAS No.: 6055-19-2), was obtained from Sigma Chemical Company, and was dissolved and diluted in sterile distilled water to stock concentrations of 1000 and 2000 µg/mL for use as the positive control in the S9-activated test system. For each positive control one dose with sufficient scorable metaphase cells was selected for analysis. The solvent for the test article was used as the solvent control at the same concentration as that found in the test article-treated groups. Complete medium or S9 reaction mixture was used in the untreated control.

MATERIALS AND METHODS

Test System

Chinese hamster ovary (CHO-K_1) cells (repository number CCL 61) were obtained from American Type Culture Collection, Rockville, MD. In order to assure the karyotypic stability of the cell line, working cell stocks were not used beyond passage 20. The freeze lot of cells was tested using the Hoechst staining procedure and found to be free of mycoplasma contamination.

The use of CHO cells has been demonstrated to be an effective method of detection of chemical clastogens (Preston et al., 1981).

Metabolic Activation System

Aroclor® 1254-induced rat liver S9 was used as the metabolic activation system. The S9 was prepared from male Sprague-Dawley rats induced with a single intraperitoneal injection of Aroclor® 1254, 500 mg/kg, five days prior to sacrifice. The S9 was batch prepared and stored at ≤-70°C until used. Each bulk preparation of S9 was assayed for sterility and its ability to metabolize 2-aminoanthracene and 7,12-dimethylbenz(α)anthracene to forms mutagenic to *Salmonella typhimurium* TA100.

Immediately prior to use, the S9 was thawed and mixed with a cofactor pool to contain 2 mM magnesium chloride, 6 mM potassium chloride, 1 mM glucose-6-phosphate, 1 mM nicotinamide adenine dinucleotide phosphate (NADP) and 20 µl S9 per milliliter medium (McCoy's 5A serum-free medium supplemented with 100 units penicillin and 100 µg streptomycin/ml, and ? mM L-glutamine).

Chromosome Aberration Assay

The chromosome aberration assay was performed using standard procedures (Evans, 1976), by exposing duplicate cultures of CHO cells to the test article as well as positive, solvent and untreated controls. For the chromosome aberration assay, CHO cells were seeded at approximately 5 x 10^5 cells/25 cm^2 flask and were incubated at 37±1°C in a humidified atmosphere of 5±1% CO_2 in air for 16-24 hours. Treatment was carried out by refeeding duplicate flasks with 5 ml complete medium (McCoy's 5A medium supplemented with 10% FBS, 100 units penicillin and 100 µg streptomycin/mL, and 2 mM L-glutamine) for the non-activated study or 5 ml S9 reaction mixture for the S9-activated study, to which was added 50 µl of dosing solution of test or control article in solvent or solvent alone. An untreated control consisting of cells in complete medium or S9 reaction mixture was also included.

In the non-activated study, the cells were exposed to the test article continuously up to cell harvest at 37±1°C in a humidified atmosphere of 5±1% CO_2 in air. Two hours prior to the scheduled cell harvest, Colcemid® was added to duplicate flasks for each treatment condition at a final concentration of 0.1 µg/mL and the flasks returned to the incubator until cell collection.

In the S9-activated study, the cells were exposed for 4 hours at 37±1°C in a humidified atmosphere of 5±1% CO_2 in air. After the exposure period, the treatment medium was removed, the cells washed with CMF-PBS, refed with complete medium and returned to the incubator. Two hours prior to the scheduled cell harvest, Colcemid® was added to duplicate flasks for each treatment condition at a final concentration of 0.1 µg/mL and the flasks were returned to the incubator until cell collection.

A concurrent toxicity test was conducted in both the non-activated and the S9-activated studies. After cell harvest an aliquot of the cell suspension was removed from each culture and counted using a Coulter counter. Cell viability was determined by trypan blue dye exclusion. The cell counts and percent viability were used to determine cell growth inhibition relative to the solvent control.

Collection of Metaphase Cells

Two hours after the addition of Colcemid®, metaphase cells were harvested for both the non-activated and S9-activated studies by trypsinization. Cells were collected approximately 12 hours after initiation of treatment in the non-activated assay and at 20 hours after treatment initiation in the S9-activated assay. The cells were collected by centrifugation at approximately 800 rpm for 5 minutes. The cell pellet was resuspended in 2-4 ml 0.075 M potassium chloride (KCl) and allowed to stand at room temperature for 4-8 minutes. The cells were collected by centrifugation, the supernatant aspirated and the cells fixed with two washes of approximately 2 ml Carnoy's fixative (methanol:glacial acetic acid, 3:1, v/v). The cells were stored overnight or longer in fixative at approximately 2-6°C.

Slide Preparation

To prepare slides, the fixed cells were centrifuged at approximately 800 rpm for 5 minutes, the supernatant was aspirated, and 1 ml fresh fixative was added. After additional centrifugation (at approximately 800 rpm for 5 minutes) the supernatant fluid was decanted and the cells resuspended to opalescence in fresh fixative. A sufficient amount of cell suspension was dropped onto the center of a glass slide and allowed to air dry overnight. Slides were identified by the study number, date prepared and the treatment condition. The dried slides were stained with 5% Giemsa, air dried and permanently mounted.

Evaluation of Metaphase Cells

Slides were coded using random numbers by an individual not involved with the scoring process. To ensure that a sufficient number of metaphase cells were present on the slides, the percentage of cells in mitosis per 500 cells scored (mitotic index) was determined for each treatment group. Metaphase cells with 20±2 centromeres were examined under oil immersion without prior knowledge of treatment groups. Whenever possible, a minimum of 200 metaphase spreads (100 per duplicate flask) were examined and scored for chromatid-type and chromosome-type aberrations. Chromatid-type aberrations include chromatid and isochromatid breaks and exchange figures such as quadriradials (symmetrical and asymmetrical interchanges), triradials, and complex rearrangements. Chromosome-type aberrations include chromosome breaks and exchange figures such as dicentrics and rings. Fragments (chromatid or acentric) observed in the absence of any exchange figure were scored as a break (chromatid or chromosome). Fragments observed with an exchange figure were not scored as an aberration but instead were considered part of the incomplete exchange. Pulverized chromosome(s), pulverized cells and severely damaged cells (≥10 aberrations) were also recorded. Chromatid and isochromatid gaps were recorded but not included in the analysis. The XY coordinates for each cell with chromosomal aberrations were recorded using a calibrated microscope stage.

Evaluation of Test Results

The toxic effects of treatment were based upon cell growth inhibition relative to the solvent-treated control and are presented for the aberration study.

The number and types of aberrations found, the percentage of structurally damaged cells (percent aberrant cells) in the total population of cells examined, and the frequency of structural aberrations per cell (mean aberrations per cell) were calculated and reported for each group. Chromatid and isochromatid gaps are presented in the data but are not included in the total percentage of cells with one or more aberrations or in the frequency of structural aberrations per cell.

Statistical analysis of the percent aberrant cells was performed using the Fisher's exact test. Fisher's test was used to compare pairwise the percent aberrant cells of each treatment group with that of the solvent control. In the event of a positive Fisher's test at any test article dose level, the Cochran-Armitage test was used to measure dose-responsiveness.

All conclusions were based on sound scientific basis; however, as a guide to interpretation of the data, the test article was considered to induce a positive response when the percentages of cells with aberrations were increased in a dose-responsive manner with one or more concentrations being statistically elevated relative to the solvent control group ($p \leq 0.05$). A significant increase at the high dose only with no dose response was considered suspect. A significant increase at one dose level other than the high dose with no dose response was considered equivocal. Test articles that did not demonstrate a statistically significant increase in aberrations were concluded to be negative.

Criteria for a Valid Test

The frequency of cells with structural chromosome aberrations in either the untreated or solvent control must be no greater than 6%. The percentage of cells with chromosome aberrations in the positive control must be statistically increased ($p \leq 0.05$, Fisher's exact test) relative to the solvent control or to the untreated control if a solvent other than water was used.

Archives

Upon completion of the final report, all raw data, reports, and stained and coded slides are maintained in the archives of Microbiological Associates, Inc., located in Rockville, Maryland.

RESULTS AND DISCUSSION

Solubility

Ethanol was determined to be the solvent of choice based on information from the Sponsor, solubility of the test article and compatibility with the target cells. The test article was soluble in ethanol at a concentration of 500 mg/ml, the maximum concentration tested.

Chromosome Aberration Assay

The dose levels and harvest times for the chromosome aberration assay were selected by the Sponsor. The dose levels tested in both the non-activated and S9-activated studies were 313, 625, 1250, 2500, and 5000 µg/mL. The test article was soluble in treatment medium at all dose levels tested. The harvest times selected for the non-activated and S9-activated studies were 12 and 20 hours after treatment initiation, respectively. In the first assay, at the 5000 µg/mL dose level in the non-activated study, a discrepancy in the mitotic indices from the A and B flasks was noted (the B flask contained metaphase cells, the A flask did not). The non-activated assay was repeated due to this discrepancy and only the data from the repeat assay is reported.

The activity of Tertiary Amyl Methyl Ether (TAME) in the induction of chromosome aberrations in CHO cells when treated at 625, 1250, 2500, and 5000 µg/ml in the absence of S9-activation is presented by treatment flask in Table 2 and summarized by group in Table 5. Toxicity (cell growth inhibition relative to the solvent control) was approximately 70% at 5000 µg/mL, the highest test concentration evaluated for structural chromosome aberrations (Table 1). Concentrations of 625, 1250, 2500 and 5000 µg/ml were evaluated for chromosome aberrations. An additional concentration of 313 µg/mL was tested as a safeguard against excessive toxicity at higher concentrations but were not required for microscopic examination. The mitotic index of the highest dose level evaluated for chromosome aberrations, 5000 µg/mL, was approximately 90% reduced, relative to the solvent control. The percentage of cells with structural aberrations in the test article-treated groups was significantly increased above that of the solvent control at dose levels 2500 and 5000 µg/mL ($p \leq 0.01$, Fisher's exact test). The Cochran-Armitage test was also positive for a dose response ($p \leq 0.05$). The percentage of damaged cells in the MMC group was 12.5% ($p \leq 0.01$, Fisher's exact test).

The activity of Tertiary Amyl Methyl Ether (TAME) in the induction of chromosome aberrations in CHO cells when treated in the presence of an S9 reaction mixture is presented by treatment flask in Table 4 and summarized by group in Table 5. Toxicity (cell growth inhibition relative to the solvent control) was approximately 48% at 2500 µg/mL, the highest test concentration evaluated for structural chromosome aberrations (Table 3). Concentrations of 313, 625, 1250 and 2500 µg/ml were evaluated for chromosome aberrations. Dose level 5000 µg/mL was not evaluated due to complete mitotic inhibition (no metaphase cells were observed). The mitotic index of the highest dose level evaluated for chromosome aberrations, 2500 µg/mL, was not reduced relative to the solvent control. The percentage of cells with structural aberrations in the test article-treated groups was statistically increased above that of the solvent control at all dose levels evaluated, 313, 625, 1250, and 2500 µg/mL ($p \leq 0.01$, Fisher's exact test). The Cochran-Armitage test was also positive for a dose response ($p \leq 0.05$). The percentage of damaged cells in the CP group was 53.5% ($p \leq 0.01$, Fisher's exact test).

CONCLUSION

The positive and negative controls fulfilled the requirements for a valid test.

Under the conditions of the assay described in this report, Tertiary Amyl Methyl Ether (TAME) was concluded to be positive in the chromosome aberration assay using Chinese hamster ovary (CHO) cells.

REFERENCES

Evans, H.J. (1976) Cytological methods for detecting chemical mutagens, in: A. Hollaender (Ed.), *Chemical Mutagens, Principles and Methods for their Detection*, vol 4. Plenum Press, New York.

Perry, P., and S. Wolff (1974) New Giemsa method for differential staining of sister chromatids, *Nature*, 251:156-158.

Preston, R.J., W. Au, M.A. Bender, J.G. Brewen, A.V. Carrano, J.A. Heddle, A.F. McFee, S. Wolff and J.S. Wassom (1981) Mammalian *in vivo* and *in vitro* cytogenetic assays: a report of the Gene-Tox Program, *Mutation Research*, 87:143-188.

TABLE 1

CONCURRENT TOXICITY TEST USING TERTIARY AMYL METHYL ETHER (TAME) IN THE ABSENCE OF EXOGENOUS METABOLIC ACTIVATION

12 HOUR CONTINUOUS TREATMENT

Treatment[1]	Replicate Flask	Cell Count ($x10^5$)	Cell Viability[2] (%)	Mean Viable Cells per Flask[3] ($x10^6$)	Cell Growth Index[4] (%)	Cell Growth Inhibition[5] (%)
Untreated	A	2.81	96%			
	B	3.08	97%	2.84	N/C	N/C
Ethanol	A	2.14	98%			
	B	2.45	96%	2.22	100%	0%
Tertiary Amyl Methyl Ether (TAME)						
313 µg/mL	A	1.93	98%			
	B	2.20	97%	2.01	90%	10%
625 µg/mL	A	2.24	98%			
	B	2.15	96%	2.13	96%	4%
1250 µg/mL	A	1.76	99%			
	B	1.79	97%	1.74	78%	22%
2500 µg/mL	A	1.40	98%			
	B	1.55	97%	1.44	65%	35%
5000 µg/mL	A	0.86	69%			
	B	0.98	75%	0.66	30%	70%
MMC,	A	2.15	97%			
0.08 µg/mL	B	2.17	98%	2.10	74%	26%
MMC,	A	2.06	96%			
0.15 µg/mL	B	2.25	94%	2.02	71%	29%

[1] CHO cells were treated in the absence of an exogenous source of metabolic activation for 12 hours at 37±1°C.
[2] Viability determined by trypan blue dye exclusion.
[3] Viable cells/flask = cell count x % viable cells, reported as mean of Flasks A and B.
[4] Growth index = (mean cells per flask treated group/mean cells per flask control group), expressed as a percentage. (Test article concentrations compared to solvent control and positive control compared to untreated control.)
[5] Cell growth inhibition = 100% - % cell growth index.

TABLE 2

CYTOGENETIC ANALYSIS OF CHO CELLS TREATED WITH TERTIARY AMYL METHYL ETHER (TAME) IN THE ABSENCE OF EXOGENOUS METABOLIC ACTIVATION

12 HOUR CONTINUOUS TREATMENT

Treatment[1,7]	Flask	Mitotic Index	Cells Scored	Aberrant Cells[2] (%)	Total Number of Structural Aberrations: Chromatid-type[3] Gaps	Breaks	Exch	Chromosome-type[4] Breaks	Dic	Ring	Severely Damaged Cells[5]	Average Aberrations Per Cell[6]
Untreated cells	A	9.0	100	2	0	2	0	0	1	0	0	0.030
	B	9.6	100	2	1	1	0	0	1	0	0	0.020
Ethanol	A	8.2	100	4	1	4	0	0	0	0	0	0.040
	B	10.4	100	2	1	1	0	2	0	0	0	0.030
Tertiary Amyl Methyl Ether (TAME)												
625 µg/mL	A	4.8	100	5	1	6	0	1	0	0	0	0.070
	B	6.2	100	5	4	4	0	0	1	0	0	0.050
1250 µg/mL	A	5.0	100	4	1	2	0	2	0	0	0	0.040
	B	5.6	100	3	2	2	0	1	0	0	0	0.030
2500 µg/mL	A	2.8	100	12	1	9	0	3	0	1	0	0.130
	B	3.0	100	8	3	6	0	2	1	0	0	0.090
5000 µg/mL	A	1.4	100	10	2	8	0	1	1	0	0	0.100
	B	0.4	52	21	0	6	0	6	0	0	0	0.231
MMC	A	5.0	100	14	0	17	1	1	0	0	0	0.190
0.08 µg/mL	B	6.6	100	11	4	9	0	3	0	0	0	0.120

1 CHO cells were treated for 12 hours at 37±1°C in the absence of an exogenous source of metabolic activation.
2 Excluding cells with only gaps.
3 Chromatid breaks include chromatid and isochromatid breaks and fragments; chromatid exchange figures include quadriradials, triradials and complex rearrangements.
4 Chromosome breaks include breaks and acentric fragments; dic, dicentric chromosome.
5 Severely damaged cells include cells with one or more pulverized chromosomes and cells with 10 or more aberrations.
6 Severely damaged cells and pulverizations were counted as 10 aberrations.
7 Additional dose level 313 µg/mL was tested but was not required for microscopic examination.

TABLE 5

CONCURRENT TOXICITY TEST USING TERTIARY AMYL METHYL ETHER (TAME) IN THE PRESENCE OF EXOGENOUS METABOLIC ACTIVATION

4 HOUR TREATMENT, 16 HOUR RECOVERY PERIOD

Treatment[1]	Replicate Flask	Cell Count ($x10^6$)	Cell Viability[2] (%)	Mean Viable Cells per Flask[3] ($x10^6$)	Cell Growth Index[4] (%)	Cell Growth Inhibition[5] (%)
Untreated	A	2.49	94%			
	B	2.73	91%	2.41	N/C	N/C
Ethanol	A	2.65	92%			
	B	2.91	96%	2.61	100%	0%
Tertiary Amyl Methyl Ether (TAME)						
313 µg/mL	A	2.23	89%			
	B	2.59	88%	2.13	82%	18%
625 µg/mL	A	2.39	96%			
	B	2.15	94%	2.16	82%	18%
1250 µg/mL	A	1.85	89%			
	B	1.86	95%	1.71	65%	35%
2500 µg/mL	A	1.48	94%			
	B	1.41	94%	1.36	52%	48%
5000 µg/mL	A	0.94	92%			
	B	0.98	94%	0.90	34%	66%
CP,	A	1.95	90%			
10 µg/mL	B	1.83	97%	1.77	73%	27%
CP,	A	1.68	85%			
20 µg/mL	B	1.57	88%	1.40	58%	42%

[1] CHO cells were treated in the presence of an exogenous source of metabolic activation for 4 hours at 37±1°C.
[2] Viability determined by trypan blue dye exclusion.
[3] Viable cells/flask = cell count x % viable cells, reported as mean of Flasks A and B.
[4] Growth index = (mean cells per flask treated group/mean cells per flask control group), expressed as a percentage. (Test article concentrations compared to solvent control and positive control compared to untreated control.)
[5] Cell growth inhibition = 100% - % cell growth index.

TABLE 4

CYTOGENETIC ANALYSIS OF CHO CELLS TREATED WITH Tertiary Amyl Methyl Ether (TAME) IN THE PRESENCE OF EXOGENOUS METABOLIC ACTIVATION

4 HOUR TREATMENT, 16 HOUR RECOVERY PERIOD

Treatment[1,7]	Flask	Mitotic Index	Cells Scored	Aberrant Cells[2] (%)	Total Number of Structural Aberrations: Chromatid-type[3] Gaps	Chromatid-type[3] Breaks	Chromatid-type[3] Exch	Chromosome-type[4] Breaks	Chromosome-type[4] Dic	Chromosome-type[4] Ring	Severely Damaged Cells[5]	Average Aberrations Per Cell[6,7]
Untreated cells	A	9.2	100	2	0	2	0	0	0	0	0	0.020
	B	9.0	100	2	0	1	0	2	0	0	0	0.030
Ethanol	A	7.2	100	3	1	2	0	0	1	0	0	0.030
	B	7.6	100	0	0	0	0	0	0	0	0	0.000
Tertiary Amyl Methyl Ether (TAME)												
313 µg/mL	A	6.8	100	10	1	7	0	4	0	1	0	0.120
	B	9.6	100	9	1	9	0	2	0	0	0	0.110
625 µg/mL	A	8.4	100	7	0	3	1	4	0	0	0	0.080
	B	10.0	100	9	1	7	1	2	0	0	0	0.100
1250 µg/mL	A	10.8	100	12	0	8	4	1	0	1	0	0.140
	B	7.8	100	20	4	7	16	2	1	1	0	0.270
2500 µg/mL	A	9.0	100	31	7	23	12	5	0	0	1	0.500
	B	7.6	100	24	3	21	8	7	0	1	1	0.470
CP	A	2.2	100	52	0	52	20	13	0	0	3	1.150
10 µg/mL	B	1.8	100	55	0	73	7	29	1	1	0	1.110

1 CHO cells were treated for 4 hours at 37±1°C in the presence of an exogenous source of metabolic activation.
2 Excluding cells with only gaps.
3 Chromatid breaks include chromatid and isochromatid breaks and fragments; chromatid exchange figures include quadriradials, triradials and complex rearrangements.
4 Chromosome breaks include breaks and acentric fragments; dic, dicentric chromosome.
5 Severely damaged cells include cells with one or more pulverized chromosomes and cells with 10 or more aberrations.
6 Severely damaged cells and pulverizations were counted as 10 aberrations.
7 Dose level 5000 µg/mL was not analyzed due to complete mitotic inhibition.

TABLE 5

SUMMARY

Treatment	S9 Activation	Treatment/Harvest Time (hrs)	Mitotic Index	Cells Scored	Aberrations Per Cell[1] (Mean ± SD)	Cells With Aberrations[2] (%)
Untreated	-	12/12	9.3	200	0.025 ± 0.186	2.0
Ethanol	-	12/12	9.3	200	0.035 ± 0.210	3.0
Tertiary Amyl Methyl Ether (TAME)						
625 µg/mL	-	12/12	5.5	200	0.060 ± 0.295	5.0
1250 µg/mL	-	12/12	5.3	200	0.035 ± 0.184	3.5
2500 µg/mL	-	12/12	2.9	200	0.110 ± 0.344	10.0**
5000 µg/mL	-	12/12	0.9	152	0.145 ± 0.371	13.8**
MMC 0.08 µg/mL	-	12/12	5.8	200	0.155 ± 0.460	12.5**
Untreated	+	4/20	9.1	200	0.025 ± 0.186	2.0
Ethanol	+	4/20	7.4	200	0.015 ± 0.122	1.5
Tertiary Amyl Methyl Ether (TAME)						
313 µg/mL	+	4/20	8.2	200	0.115 ± 0.377	9.5**
625 µg/mL	+	4/20	9.2	200	0.090 ± 0.320	8.0**
1250 µg/mL	+	4/20	9.3	200	0.205 ± 0.543	16.0**
2500 µg/mL	+	4/20	8.3	200	0.485 ± 1.220	27.5**
CP 10 µg/mL	+	4/20	2.0	200	1.135 ± 1.637	53.5**

[1] Severely damaged cells were counted as 10 aberrations.
[2] *, $p \leq 0.05$, **, $p \leq 0.01$; Fisher's exact test.

APPENDIX I

Historical Control Data

IN VITRO MAMMALIAN CYTOGENETIC TEST USING CHINESE HAMSTER OVARY (CHO) CELLS

HISTORICAL CONTROL VALUES STRUCTURAL ABERRATIONS 1994-1996

NON-ACTIVATED TEST SYSTEM

Historical Values	Aberrant Cells		
	Untreated Control	Solvent Control[1]	Positive Control[2]
Mean	1.0%	1.1%	27.3%
Standard Deviation	1.0%	1.1%	16.4%
Range	0.0% to 5.5%	0.0% to 5.5%	7.5% to 94.0%

S9-ACTIVATED TEST SYSTEM

Historical Values	Aberrant Cells		
	Untreated Control	Solvent Control[1]	Positive Control[3]
Mean	1.4%	1.3%	42.7%
Standard Deviation	1.3%	1.2%	24.6%
Range	0.0% to 5.5%	0.0% to 6.0%	7.5% to 100.0%

[1]Solvents include water, saline, dimethylsulfoxide, ethanol, acetone, non-standard solvents and Sponsor-supplied vehicles.
[2]Positive control for non-activated studies, triethylenemelamine (TEM, 0.25-0.5 µg/mL,) N-methyl-N'-nitro-N-nitrosoguanidine (MNNG, 0.75-2 µg/mL), and Mitomycin C (MMC, 0.08-0.15 µg/mL).
[3]Positive control for S9-activated studies, cyclophosphamide (CP, 10-50 µg/mL), and benzo(α)pyrene, (B[α]P, 30 µg/mL).

APPENDIX II

Study Protocol

PROTOCOL AMENDMENT 1

QA cc 2-25-97 APPROVED

SPONSOR: American Petroleum Institute

TEST ARTICLE I.D.: Tertiary Amyl Methyl Ether (TAME)

MA STUDY NO: G96CJ24.330 (Protocol No.: SPGT330)

SPONSOR PROJECT NO.: HES3251-HF-08200TEST

PROTOCOL TITLE: Chromosome Aberrations in Chinese Hamster Ovary (CHO) Cells

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1. LOCATION: Page 2 of 7, § 7.2 Dose Levels;

AMENDMENT: Add the following sentence to this section: "Whenever possible, the high dose for the chromosome aberration assay will be selected to give at least 50% toxicity (cell growth inhibition relative to the solvent). At least three additional dose levels will be evaluated for aberrations in the chromosome aberration assay."

REASON FOR THE AMENDMENT: To define the number of dose levels evaluated for aberrations in the assay.

APPROVALS:

Ramadevi Gud — STUDY DIRECTOR — 2/25/97 DATE

Robert A. Sheldon — SPONSOR REPRESENTATIVE — 2/28/97 DATE

Chromosome Aberrations in Chinese Hamster Ovary (CHO) Cells

1.0 PURPOSE

The purpose of this study is to evaluate the clastogenic potential of a test article based upon its ability to induce chromosome aberrations in Chinese hamster ovary (CHO) cells.

2.0 SPONSOR

2.1 Name: **American Petroleum Institute**

2.2 Address: **1220 L Street, Northwest**
Washington, D.C. 20005

2.3 Representative: **Richard A. Rhoden, Ph.D.**

2.4 Sponsor Project #: **HES3251-HF-08200TEST**

3.0 IDENTIFICATION OF TEST AND CONTROL SUBSTANCES

3.1 Test Article: **Tertiary Amyl Methyl Ether (TAME)**

3.2 Controls:
Untreated: Untreated Cells
Solvent: Test Article Solvent
Positive: Mitomycin C (MMC), Cyclophosphamide (CP)

3.3 Determination of Strength, Purity, etc.

The Sponsor will be directly responsible for determination and documentation of the analytical purity and composition of the test article and the stability and strength of the dosing solutions.

3.4 Test Article Retention Sample

The retention of a reserve sample of the test article will be the responsibility of the Sponsor.

4.0 TESTING FACILITY AND KEY PERSONNEL

4.1 Name: **Toxicology Testing Facility**
Microbiological Associates, Inc.

4.2 Address: **9630 Medical Center Drive**
Rockville, MD 20850

4.3 Study Director: **Ramadevi Gudi, Ph.D.**

5.0 TEST SCHEDULE

5.1 Proposed Experimental Initiation Date: 2/24/97

5.2 Proposed Experimental Completion Date: 4/16/97

5.3 Proposed Report Date: 4/30/97

6.0 TEST SYSTEM

The CHO-K_1 cell line is a proline auxotroph with a modal chromosome number of 20 and a population doubling time of 10-14 hours. CHO-K_1 cells were obtained from the American Type Culture Collection (repository number CCL 61), Rockville, MD. This system has been demonstrated to be sensitive to the clastogenic activity of a variety of chemicals (Preston et al., 1981).

7.0 EXPERIMENTAL DESIGN AND METHODOLOGY

The chromosome aberration assay will be conducted using standard procedures (Evans, 1976), by exposing cultures of CHO cells to five concentrations of the test article as well as to positive, untreated, and solvent controls. In the non-activated test system, treatment will be continuous up to the time of cell collection of 12 hours after the initiation of the treatment; in the S9 activated test system, exposure will be for 4 hours. The dividing cells will be arrested in metaphase and harvested for microscopic evaluation of chromosome aberrations at approximately 12 hours after the initiation of treatment in the non-activated studies and 20 hours after the initiation of treatment in the S9-activated studies. The clastogenic potential of the test article will be measured by its ability to increase structural chromosome aberrations in a dose-responsive manner when compared to the solvent control group.

7.1 Test article solvent

Ethanol (CAS 64-17-5) will be used as the test article solvent.

7.2 Dose Levels

Dose levels for the cytogenetics assay will be 313, 625, 1250, 2500 and 5000 µg/ml in the presence and absence of S9 activation.

7.3 Frequency and Route of Administration

Target cells will be treated for 12 hours in the absence of S9 and for 4 hours in the presence of S9 by incorporation of the test article-solvent mixture into the treatment medium. This technique has demonstrated to be an effective method of detection of chemical clastogens in this test system.

7.4 Activation System

Aroclor 1254-induced rat liver S9 will be used as the metabolic activation system. The S9 will be prepared from male Sprague-Dawley rats induced with a single intraperitoneal injection of Aroclor 1254, 500 mg/kg, five days prior to sacrifice. The S9 will be batch prepared and stored frozen at approximately -70°C until used.

Immediately prior to use, the S9 will be thawed and mixed with cofactors to contain 2 mM magnesium chloride ($MgCl_2$), 6 mM potassium chloride (KCl), 1 mM glucose-6-phosphate, 1 mM nicotinamide adenine dinucleotide phosphate (NADP) and 20 µl S9 per ml serum free medium.

7.5 Controls

7.5.1 Untreated Control

Untreated cells will be used as the untreated control.

7.5.2 Solvent Control

The solvent for the test article will be used as the solvent control. For solvents other than water, physiological buffer or culture medium, the final concentration in treatment medium will not exceed 1%.

7.5.3 Positive Controls

Mitomycin C will be used at a concentration within 0.05-0.3 µg/ml as the positive control in the non-activated study. Cyclophosphamide will be used at a concentration within 10-50 µg/ml as the positive control in the S9-activated study.

7.6 Preparation of Target Cells

Exponentially growing CHO-K_1 cells will be seeded in complete medium (McCoy's 5A medium containing 10% fetal bovine serum, 2 mM L-glutamine, 100 units penicillin/ml and 100 µg streptomycin/ml) for each treatment condition at approximately 5×10^3 cells/25 cm^2 flask. The flasks will be incubated at 37 ± 1°C in a humidified atmosphere of 5 ± 1% CO_2 in air for 16-24 hours.

7.7 Identification of Test System

Using a permanent marking pen, the treatment flasks will be identified by the MA study number and a code system to designate the treatment condition and test phase.

7.8 Treatment of Target Cells

Treatment will be carried out in duplicate by refeeding the flasks with 5 ml complete medium for the non-activated exposure or 5 ml S9 reaction mixture for the S9-activated exposure, to which will be added 50 µl of dosing solution of test or control article in solvent or solvent alone. Larger volumes of dosing solution may be used if water, physiological buffer, or medium is used as the solvent. An untreated control consisting of cells in complete medium or S9 reaction mixture will also be included.

In the non-activated study, the cells will be treated for 12 hours at 37 ± 1°C in a humidified atmosphere of 5 ± 1% CO_2 in air. Treatment will continue until collection of metaphase cells.

In the S9-activated study, the cells will be treated for 4 hours at 37 ± 1°C in a humidified atmosphere of 5 ± 1% CO_2 in air. After the treatment period, the treatment medium will be aspirated, the cells washed with phosphate buffered saline, refed with complete medium and returned to the incubator.

A concurrent toxicity test will be conducted in both the non-activated and the S9-activated studies. After cell harvest an aliquot of the cell suspension will be removed from each culture and counted using an automatic cell counter. Cell viability will be determined by trypan blue dye exclusion. The cell counts and percent viability will be used to determine cell growth inhibition relative to the solvent control.

7.9 Collection of Metaphase Cells

Cells will be collected approximately 12 hours after initiation of treatment for non-activated studies and 20 hours after initiation of treatment for S9-activated studies. Two hours prior to harvest, Colcemid® will be added to the cultures at a final concentration of 0.1 µg/ml.

Cells will be harvested by trypsinization, collected by centrifugation and an aliquot will be removed for counting using an automatic cell counter and trypan blue dye exclusion. The remainder of the cells will be swollen with 0.075M KCl, washed with two consecutive changes of fixative (methanol:glacial acetic acid, 3:1 v/v), capped and stored overnight or longer at approximately 2-6°C. The cell counts and percent viability will be used to determine cell growth inhibition relative to the solvent control. To prepare slides, the cells will be collected by centrifugation and resuspended in fresh fixative. The suspension of fixed cells will be applied dropwise onto a glass microscope slide and air-dried. The slide will be identified by the experiment number, treatment condition and date. The slides will then be stained with Giemsa and permanently mounted.

7.10 Scoring for Metaphase Aberrations

To ensure that a sufficient number of metaphase cells are present on the slides, the percentage of cells in mitosis per 500 cells scored (mitotic index) will be determined for each treatment group. Slides will be coded using random numbers by an individual not involved with the scoring process. Metaphase cells with 20 ± 2 centromeres will be examined under oil immersion without prior knowledge of treatment groups. Whenever possible, a minimum of 200 metaphase spreads from each dose level (100 per duplicate flask) will be examined and scored for chromatid-type and chromosome-type aberrations. Chromatid-type aberrations include chromatid and isochromatid breaks and exchange figures such as quadriradials (symmetrical and asymmetrical interchanges), triradials, and complex rearrangements. Chromosome-type aberrations include chromosome breaks and exchange figures such as dicentrics and rings. Fragments (chromatid or acentric) observed in the absence of any exchange figure will be scored as a break (chromatid or chromosome). Fragments observed with an exchange figure will not be scored as an aberration but will be considered part of the incomplete exchange. Pulverized chromosome(s), pulverized cells and severely damaged cells (≥ 10 aberrations) will also be recorded. Chromatid and isochromatid gaps will be recorded but not included in the analysis. The XY coordinates for each cell with a structural aberration will be recorded using a calibrated microscope stage. The mitotic index will be recorded as the percentage of cells in mitosis per 500 cells counted.

8.0 CRITERIA FOR DETERMINATION OF A VALID TEST

8.1 Untreated and Solvent Controls

The frequency of cells with structural chromosome aberrations in either the untreated or solvent control must be no greater than 6%.

8.2 Positive Control

The percentage of cells with aberrations must be statistically increased ($p \leq 0.05$, Fisher's exact test) relative to the solvent control or to the untreated control if a solvent other than water was used.

9.0 EVALUATION OF TEST RESULTS

The number and types of aberrations found, the percentage of structurally damaged cells in the total population of cells examined, and the mean aberrations per cell will be calculated and reported for each treatment group. Chromatid and isochromatid gaps are presented in the data but are not included in the total percentage of cells with one or more aberrations or in the frequency of structural aberrations per cell. Statistical analysis of the percentage of aberrant cells will be performed using the Fisher's exact test. The Fisher's test will be used to compare pairwise the percent aberrant cells of each treatment group with that of the solvent control. In the event of a positive Fisher's exact test at any test article dose level, the Cochran-Armitage test will be used to measure dose-responsiveness.

All conclusions will be based on sound scientific basis; however, as a guide to interpretation of the data, the test article will be considered to induce a positive response when the percentage of cells with aberrations is increased in a dose-responsive manner with one or more concentrations being statistically significant ($p \leq 0.05$). A significant increase at the high dose only with no dose response will be considered suspect. A significant increase at one dose level other than the high dose with no dose response will be considered equivocal. Test articles not demonstrating a statistically significant increase in aberrations will be concluded to be negative.

10.0 REPORT

A report of the results of this study will be prepared by Microbiological Associates, Inc. and will accurately describe all methods used for generation and analysis of the data.

Results presented will include, but not be limited to:

- cells used
- test conditions: composition of medium, CO_2 concentration, incubation time, concentration of test article and rationale for selection of concentration, duration of treatment, duration of treatment with and concentration of Colcemid®, type of metabolic activation system used, positive, untreated, and solvent controls
- number of cell cultures
- mitotic index and number of metaphases analyzed (method for determination; data given separately for each culture)
- cell growth inhibition relative to the solvent control
- criteria for scoring aberrations
- type and number of aberrations, given separately for each treated and control culture
- historical control data

11.0 RECORDS AND ARCHIVES

Upon completion of the final report, all raw data and reports will be maintained by the Quality Assurance Unit of Microbiological Associates, Inc., Rockville, MD, in accordance with the relevant Good Laboratory Practice Regulations.

12.0 REGULATORY REQUIREMENTS/GOOD LABORATORY PRACTICE

This protocol was designed to fulfill OECD Guideline 473 (Genetic Toxicology: *In Vitro* Mammalian Cytogenetic Test), May, 1983; and EPA Health Effects Testing Guidelines, Subpart 798.5375 (*In Vitro* Mammalian Cytogenetics), Fed. Register, vol. 50, September, 1985 with revisions Fed. Register, vol. 52, May, 1987.

This study will be performed in compliance with the provisions of the Good Laboratory Practice Regulations For Non-clinical Laboratory Studies.

Will this study be submitted to a regulatory agency? yes

If so, to which agency or agencies? EPA

Unless arrangements are made to the contrary, unused dosing solutions will be disposed of following administration to the test system and all residual test article will be disposed of following finalization of the report.

13.0 REFERENCES

Evans, H.J. (1976) Cytological methods for detecting chemical mutagens, in: A. Hollaender (Ed.), Chemical Mutagens, Principles and Methods for their Detection, vol. 4. Plenum Press, New York, NY.

Perry, P. and S. Wolff (1974) New Giemsa method for differential staining of sister chromatids, Nature, 251: 156-158.

Preston, R.J., W. Au, M.A. Bender, J.G. Brewen, A.V. Carrano, J.A. Heddle, A.F. McFee, S. Wolff and J.S. Wassom (1981) Mammalian in vivo and in vitro cytogenetic assays: a report of the Gene-Tox Program, Mutation Research, 87:143-188.

14.0 APPROVAL

Richard A. Rhoden 1/8/97

SPONSOR REPRESENTATIVE DATE

Richard A. Rhoden, Ph.D.

(Print or Type Name)

Ramadevi Gudi 1/15/97

MA STUDY DIRECTOR DATE